

# **Multivesicular bodies mediate long-range retrograde NGF-TrkA signaling**

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## Abstract

Target-derived nerve growth factor (NGF), by activating its receptor TrkA on distal axons, supports survival, growth and maintenance of connectivity of postganglionic sympathetic neurons and small-diameter primary sensory neurons of the peripheral nervous system (PNS). Long-range retrograde NGF signals are propagated by TrkA<sup>+</sup> signaling endosomes, yet signaling endosome properties and their mechanisms of formation, axonal trafficking and signaling are poorly understood. The prevailing view is that Rab5<sup>+</sup> early endosomes mediate retrograde NGF signals. In this study, we showed that ultrastructurally- and molecularly-defined multi-vesicular bodies (MVBs), but not early endosomes, are major carriers of retrograde NGF signals. Importantly, retrogradely transported TrkA<sup>+</sup> MVBs in cell bodies associate with key effectors of TrkA, suggesting that they are *bona fide* signaling endosomes. Moreover, we found that, Rab7, a key regulator of MVB function, mediates survival and synaptogenesis of sympathetic neurons *in vivo*. Rab7 and its effector protein RILP, are associated with TrkA MVBs and are required for retrograde TrkA transport and survival *in vitro*. Remarkably, in contrast to MVBs carrying a variety of non-TrkA cargoes, retrogradely transported TrkA<sup>+</sup> MVBs that arrive in cell bodies evolve into TrkA<sup>+</sup> single-membrane vesicles (SVs) that evade lysosomal fusion. This novel population of TrkA SVs are signaling competent and therefore can contribute to NGF signal transduction. Molecularly, TrkA SVs are not Rab5<sup>+</sup>. Instead, a significant portion of TrkA SVs are associated with Vps35, a key component of the retromer complex. Lastly, TrkA kinase activity associated with retrogradely transported TrkA<sup>+</sup> MVBs critically determines TrkA<sup>+</sup> endosome evolution and fate. Thus, MVBs uniquely mediate long-range axonal transport of TrkA and serve as essential signaling and sorting platforms in the cell soma, and MVB cargoes dictate their vesicular fate.

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## **Chapter 1: Introduction**



## **The neurotrophins: target-derived trophic factors**

The establishment of functional neural circuits requires proper communication between the nervous system and the periphery. The development and maintenance of connectivity of neurons in the peripheral nervous system (PNS) are critically dependent on target-derived cues released at the end organ that they innervate. The neurotrophin family of growth factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4 (NT4), are the best-characterized target-derived neurotrophic growth factors. Through binding to and activating their respective receptors (NGF to TrkA, BDNF and NT4 to TrkB, NT3 predominantly to TrkC), these factors play essential and instructive roles in neuronal survival, axonal growth, neuronal subtype specification, synapse formation, and maintenance (Cosker et al., 2008; Harrington and Ginty, 2013; Huang and Reichardt, 2001; Luo et al., 2007; Miller and Kaplan, 2001; Sharma et al., 2010).

Key to the development and function of PNS is that the amount of neuronal innervation has to match the size and demands of the end organ, a process termed “systems matching” (Levi-Montalcini, 1987). The first glimpse of how systems matching is achieved came from seminal discoveries done by Victor Hamburger and Rita Levi-Montalcini (Hamburger and Levi-Montalcini, 1949). In a series of experiments conducted on chick embryos, they demonstrated that removal of limb bud led to dramatic increase of cell death of developing sensory and motor neurons. These observations formed the basis of the “neurotrophic factor hypothesis” (Oppenheim, 1989).

The neurotrophic factor hypothesis posits that, during development, neurons are over-produced and competing for limiting amount of survival factors that is released within the peripheral tissues. Neurons that have reached the correct target region and received adequate

amount of neurotrophic factors will survive; those that have either derailed or received too little trophic cues will die (Oppenheim, 1989). This developmental process ultimately ensures a balance between the number of surviving neurons and the size and demands of the target field. This also provides a plausible explanation for the seemingly puzzling and inefficient developmental sequence of neuronal over production followed by massive cell death (Barde et al., 1989; Buss and Oppenheim, 2004).

Following these earlier studies, subsequent purification of the neurotrophins and identification and cloning of their receptors, Trk receptor tyrosine kinases, laid the foundation for understanding the cellular and molecular functions of these factors and their essential roles in different parts of the nervous system. Knock-out mouse models have proven to be invaluable for not only confirming earlier studies but also greatly extending our knowledge of the physiological roles of neurotrophins (Snider, 1994). First of all, in each of the neurotrophin- and Trk-knock out mice, PNS neurons suffer from increased apoptosis and thus exhibit a reduction in cell counts (Snider, 1994). This underscores the critical requirement of neurotrophins and their receptors for neuronal survival during development. Secondly, different neurotrophin gene knockout result in distinct patterns of neuronal cell loss, indicating that they are differentially required for survival of different neuronal populations. For example, NGF and TrkA knockout have reduced cell numbers in postganglionic sympathetic neurons, trigeminal ganglia sensory neurons and small-diameter dorsal root ganglia (DRG) sensory neurons (Crowley et al., 1994; Smeyne et al., 1994); BDNF and TrkB knockout affect vestibular ganglia neurons, nodose ganglia neurons and a subset of non-TrkA DRG neurons (Ernfors et al., 1994a; Klein et al., 1993); NT3 and TrkC knockout affect select classes of large-diameter DRG neurons including proprioceptors (Ernfors et al., 1994b; Klein et al., 1994). Thirdly, differential phenotypes exhibited between gene

knockout of a neurotrophic factor and its cognate receptor highlight cognate ligand-receptor pairing. For example, while sympathetic neurons die in NT3 null mice, they are largely spared in TrkC null mice, suggesting a TrkC-independent signaling mechanisms of NT3 for survival of sympathetic neurons (Ernfors et al., 1994b; Klein et al., 1994). Indeed, in this context, NT3 acts via TrkA, not TrkC, to serve as an intermediate-target derived cue for sympathetic neuron axon growth (Kuruvilla et al., 2004). Lastly, by genetically preventing apoptosis in neurotrophin knockout mice, one can study the roles of neurotrophins in neuronal development beyond the stage of survival. In NGF<sup>-/-</sup>; Bax<sup>-/-</sup> mice, sympathetic neurons do not undergo apoptosis (Middleton and Davies, 2001; Patel et al., 2000a). However, in these animals, final target innervation of sympathetic axons in various peripheral tissues is compromised, as is synapse formation between pre- and post-ganglionic sympathetic neurons (Glebova and Ginty, 2004; Sharma et al., 2010). These observations strongly indicate that, in addition to survival, NGF is critical for target innervation and synaptogenesis.

### **Long-range retrograde neurotrophin signaling**

NGF is the first described and prototypic neurotrophin. Acting via its cognate receptor, TrkA, NGF is essential for survival, growth and maintenance of connectivity in select classes of PNS neurons (Huang and Reichardt, 2001). A remarkable feature of NGF signaling, and perhaps for other neurotrophic factors as well, is that, in order to elicit its actions on developing PNS neurons, NGF-TrkA signals must be propagated retrogradely from the axon terminal to the soma, covering a distance that is literally thousands of times the width of the cell body (Cosker et al., 2008; Howe and Mobley, 2005).

How do NGF signals, initiated in distal axons, instruct neuronal survival? The in vitro compartmentalized culture system provides an excellent means to study retrograde NGF

signaling, trafficking and survival in a spatially and temporally controlled manner (Campenot, 1977; Taylor et al., 2005). In such a device, distal axons and cell bodies are fluidically separated. Application of NGF exclusively to the distal axon compartment thus recapitulates the target-derived nature of NGF in vivo. Indeed, NGF applied exclusively to distal axons of sympathetic neurons and TrkA<sup>+</sup> sensory neurons grown compartmentalized cultures is sufficient to support their survival (Campenot, 1977; MY and DDG, unpublished observations, see Chapter 3). Therefore, this system enables identification of pathways activated by retrograde NGF signaling and direct assessment of their contributions to retrograde NGF-dependent neuronal survival.

NGF application to distal axons leads to dimerization and autophosphorylation of TrkA receptors expressed on the axonal plasma membrane. Then, three major signaling cascades are activated downstream of NGF-TrkA: ERK1/2, PI3K and PLC $\gamma$  pathways (Huang and Reichardt, 2001). Importantly, TrkA phosphorylation and activation of these effector pathways in distal axons are required for neuronal survival (Atwal et al., 2000; Kuruvilla et al., 2000; Riccio et al., 1997; Watson et al., 2001). Activation of NGF-TrkA signaling pathways support cell survival by promoting transcription of pro-survival genes and by inhibiting pro-apoptosis pathways such as translocation of BAX to mitochondria (Pazyra-Murphy et al., 2009; Putcha et al., 1999; Riccio et al., 1999). One key transcription factor that mediates the pro-survival pathway downstream of NGF-TrkA signaling is CREB, partly by upregulation of Bcl-2 expression (Riccio et al., 1999). Furthermore, NGF-TrkA signaling activates a feedback genetic circuit which enhances self-sensitization to NGF and produces paracrine apoptotic signals to neighboring neurons (Deppmann et al., 2008).

Target-derived NGF signals influence the development of neurons beyond promotion of cell survival. Several lines of evidence, both in vivo and in vitro, suggest that NGF signaling

supports axonal growth, synapse formation and neuronal subtype specification and maturation (Harrington and Ginty, 2013). First, in compartmentalized cultures, NGF application to distal axons support local axon extension, whereas removal of NGF in the distal axon compartment leads to axon retraction, suggesting that NGF can act locally to cause extension (Campenot, 1977). In agreement with this, sympathetic neurons from NGF<sup>-/-</sup>; Bax<sup>-/-</sup> mice, while alive, fail to fully innervate target tissues, indicating a defect of axonal growth and branching (Glebova and Ginty, 2004). Second, NGF<sup>-/-</sup>; Bax<sup>-/-</sup> mice exhibit a dramatic loss of synapses in sympathetic ganglia (Sharma et al., 2010). In vitro, NGF withdrawal from the distal axon compartment disrupts post-synaptic density (PSD) formation in dendrites of sympathetic neurons in the cell body compartment, while re-addition of NGF exclusively to distal axons leads to appearance of PSD in dendrites, suggesting retrograde NGF is necessary and sufficient for PSD formation (Sharma et al., 2010). Moreover, inhibition of TrkA kinase activity and thus NGF-TrkA signaling locally in the somatic/dendritic region disrupts synaptogenesis, both in vivo and in vitro (Lehigh et al., 2017; Sharma et al., 2010). Lastly, NGF instructs specification of both peptidergic and non-peptidergic nociceptors via distinct genetic programs (Chen et al., 2006; Huang et al., 2015; Luo et al., 2007; Patel et al., 2000b). Target-derived NGF is required for the expression of CGRP, a key molecular determinant of the peptidergic nociceptor (Patel et al., 2000b). On the other hand, NGF promotes specification of non-peptidergic nociceptors by controlling expression of two key factors, Ret and CBF $\beta$  (Huang et al., 2015; Luo et al., 2007).

Taken together, target-derived NGF signals play essential roles in various aspects of neuronal development, including survival, axonal growth and target field innervation, neuronal subtype specification and, for sympathetic neurons, synapse formation.

## **Mechanisms of retrograde signaling**

Target-fields control survival and development of PNS neurons via long-range retrograde neurotrophic signaling. How does NGF, secreted in the periphery, transmit essential survival signals from the distal axon terminal to the cell soma? What is the biochemical and molecular nature of retrograde NGF signal? NGF injection in target fields of sympathetic neurons in vivo and NGF application in distal axons of compartmentalized cultures demonstrated that NGF itself is retrogradely transported (Claude et al., 1982a; Johanson et al., 1995; Sandow et al., 2000). Moreover, phosphorylated TrkA (P-TrkA) was observed in rat sciatic nerves in vivo, and NGF application in distal axons led to appearance of P-TrkA in cell bodies in vitro, indicating that P-TrkA, the activated TrkA, is also retrogradely transported (Bhattacharyya et al., 2002; Riccio et al., 1997). In addition, TrkA co-immunoprecipitated with distal axon-derived NGF in distal axons and cell bodies, suggesting that NGF and TrkA co-transport as a complex (Tsui-Pierchala and Ginty, 1999). On the other hand, prevention of NGF internalization in distal axons by crosslinking or dynamin inhibition compromised retrograde NGF signaling (Riccio et al., 1997; Ye et al., 2003). Therefore, the NGF-TrkA complex is retrogradely transported from distal axons to cell bodies and the physical translocation of the complex underlies transduction of retrograde NGF signals.

### *The 'signaling endosome' hypothesis*

These findings, taken together, support a model in which retrograde signal transmission is through the assembly of NGF-TrkA signaling endosomes (Howe and Mobley, 2005). The signaling endosome model postulates that the internalized NGF/TrkA complex at axon terminals is actively sorted into vesicles, a subset of which become mature, signaling-competent vesicles that undergo long-distance retrograde transport. By recruitment of TrkA effectors, these

organelles function as signaling platforms that initiate, amplify, maintain, and propagate retrograde signals.

Numerous studies have provided evidence that supports different aspects of this model, including formation, maturation, trafficking and signaling of NGF-TrkA signaling endosomes. First, after NGF stimulation, P-TrkA was found in clathrin-coated vesicles and was associated with key downstream signaling molecules including P-ERK1/2, PI3K and PLC $\gamma$  (Delcroix et al., 2003). Blockade of ligand-receptor internalization by expression of a dominant-negative dynamin mutant in sympathetic neurons inhibited retrograde NGF/TrkA transport and survival (Ye et al., 2003). Following internalization, NGF and TrkA were found to be associated with various types of endosomal structures in axons, cell soma and dendrites in vivo and in vitro, suggesting an endosome-based mechanism of retrograde transport and signaling (See next section). Transmission of retrograde NGF signals is dependent on dynein, a motor protein that mediates microtubule minus-end directed transport (Heerssen et al., 2004).

#### *The nature of retrograde TrkA signaling endosomes*

Despite increasing evidence that supports the signaling endosome hypothesis, the identity of the NGF/TrkA signaling endosome has been controversial. In a canonical pathway of ligand-receptor endocytosis, the internalized complex is first sorted into early endosomes (EE), and then progresses into multivesicular bodies (MVB) by homotypic fusion and formation of intraluminal vesicles. MVBs then mature into late endosomes (LE), which can be fused with lysosomes (LE) where receptors and other cargo are degraded. Along the vesicular maturation pathway, receptors can be re-inserted to the plasma membrane via recycling endosomes (Zerial and McBride, 2001). A long-standing, canonical view is that retrograde NGF/TrkA signaling endosomes are early endosomes because, like other ligand/receptor complexes, internalized NGF/TrkA complexes are

sorted into early endosomes and the membrane topology of receptors in early endosome-type vesicles can, in principle, enable signal propagation within the cytoplasm. A few studies using different experimental paradigms have implicated the early endosome as a retrograde NGF signal carrier. Following NGF injection in the foot, NGF was found associated with the small GTPase Rab5, a molecular marker and master regulator of early endosomes, in cell bodies of DRG sensory neurons (Delcroix et al., 2003). Furthermore, in an *ex vivo* sciatic nerve preparation, Rab5 was enriched in purified axoplasmic fractions that contained retrograde  $^{125}\text{I}$ -NGF. Also, TrkA was found associated with single-membrane vesicles labeled by Rab5 (Delcroix et al., 2003). In compartmentalized cultures of dorsal root ganglion (DRG) neurons, retrogradely transported NGF conjugated to quantum dots was colocalized with Rab5 (Cui et al., 2007).

However, despite the widely-accepted view that signaling endosomes are early endosomes, late endosomes (LEs) and multi-vesicular bodies (MVBs) have been observed to contain retrogradely transported cargoes in other experimental paradigms. Evidence supporting the late endosome as the NGF/TrkA signaling endosome comes from a study utilizing a tetanus toxin derivative (Deinhardt et al., 2006). In cultured DRG and motor neurons, internalized tetanus toxin was sorted into Trk<sup>+</sup> vesicles. Furthermore, it was associated with both Rab5 and Rab7 (a small GTPase enriched in LEs) in axons, but only the Rab7<sup>+</sup> vesicles underwent long-range retrograde transport (Deinhardt et al., 2006). In addition, in compartmentalized sympathetic neuron cultures,  $^{125}\text{I}$ -NGF applied to distal axons was found predominantly in MVBs and lysosomes, based on ultrastructural analysis (Claude et al., 1982b). In related work, gold-labeled NGF injected into the anterior chamber of the eye accumulated in MVBs in axons near sympathetic ganglia (Sandow et al., 2000). Also, P-TrkA was observed in multivesicular structures in the rat sciatic nerve (Bhattacharyya et al., 2002). However, BDNF injected into the



tongue did not appear associated with MVBs or any other ultrastructurally-defined endosome type in hypoglossal motor neurons (Altick et al., 2009). Moreover, a key conceptual challenge to the idea that MVBs are TrkA signal carriers, however, is the lack of a plausible mechanism to explain how signals emanating from NGF/TrkA complexes encapsulated within intraluminal vesicles of MVBs are transmitted to the cytoplasm (Howe and Mobley, 2005; Sandow et al., 2000). Thus, while several different endosomal compartments have emerged as candidate retrograde NGF transport carriers, possibly due to the different means used to visualize endosomes, different preparations, and the use of different cell types, the prevailing view is that early endosomes are retrograde TrkA<sup>+</sup> signaling endosomes.

#### *Mechanisms of signaling endosome formation, maturation, trafficking and disassembly*

Our understanding of the molecular mechanisms that underlie formation, maturation, trafficking and disassembly of signaling endosomes is largely based on pharmacological approaches. In compartmentalized neuronal cultures, inhibition of TrkA kinase activity or PI3K activity, but not RAS-MAPK activity, in distal axons, blocks retrograde NGF-TrkA transport and survival (Kuruvilla et al., 2000; Riccio et al., 1997, 1999). Furthermore, active TrkA, PI3K and RAS-MAPK in cell bodies are all required for neuronal survival (Kuruvilla et al., 2000). On the other hand, TrkA kinase activity and PI3K activity are dispensable for maintenance of retrograde NGF-TrkA transport after initiation (Kuruvilla et al., 2000; Ye et al., 2003). In addition, TrkA kinase activity and PI3K activity in distal axons and TrkA and MAPK activity in the cell body/dendrite compartment are required for retrograde NGF-dependent PSD formation in dendrites (Lehigh et al., 2017; Sharma et al., 2010).

Despite increasing knowledge of retrograde signaling, key questions remain: How do retrograde TrkA signaling endosomes become transport competent and undergo long-range

axonal movement and how do they faithfully transduce retrograde NGF signals in cell bodies? What are the effector proteins downstream of NGF-TrkA signaling that control signaling endosome formation, maturation and trafficking? Purification of TrkA endosomes followed by proteomic analysis has proven to be a useful, unbiased and high throughput means of identifying signaling endosome components and may provide novel insights into the endosome-based mechanisms of retrograde NGF signaling and survival. Indeed, one such study revealed the importance of actin dynamics in retrograde TrkA transport. Purified TrkA endosomes have actin depolymerization activity (Harrington et al., 2011). Two proteins that are responsible for this activity, Rac1 and cofilin, were found associated with TrkA endosomes and mediate actin disassembly in distal axons, which is a prerequisite for retrograde TrkA transport and survival (Harrington et al., 2011). Another newly identified TrkA signaling endosome component is Coronin-1 (Suo et al., 2014). Coronin-1 is recruited to retrograde TrkA endosomes specifically in cell bodies and prevents lysosomal fusion of TrkA endosomes. Coronin-1 is dispensable for retrograde TrkA transport but is necessary for P-CREB accumulation in cell bodies and retrograde NGF-dependent neuronal survival (Suo et al., 2014).

In this dissertation, I identified the ultrastructural and molecular nature of retrograde NGF-TrkA signaling endosomes in sympathetic neurons, using a combination of mouse genetics, in vivo and in vitro imaging analyses, electron microscopy and signaling experiments. I also identified novel TrkA endosome constituent proteins that are essential for retrograde TrkA trafficking, signaling and survival. Furthermore, I characterized maturation and membrane dynamics of retrograde TrkA endosomes in distal axons and cell bodies that underlie transport and signaling of TrkA endosomes. In addition, I tested the roles of TrkA kinase activity within endosomes in TrkA endosome maturation and turnover. My findings uncovered new mechanisms

of how endosomes and endosomal proteins mediate growth factor trafficking and signaling in neurons and hopefully will provide new insights into how target fields communicate with the nervous system and control survival, growth and connectivity of developing PNS neurons.

**Chapter 2: Multivesicular bodies convey long-range retrograde NGF  
signals from distal axons to cell bodies**

## Introduction

Target fields support development and maintenance of neurons in the peripheral nervous system via long-range retrograde signals propagated from distal axons to cell soma. The prototypical target-derived neurotrophic factor, nerve growth factor (NGF), orchestrates development of sympathetic neurons and small diameter sensory neurons. Through binding to and activating its receptor TrkA, NGF promotes neuronal survival, axonal growth and target field innervation, neuronal subtype specification and, for sympathetic neurons, synapse formation and maintenance (Cosker et al., 2008; Harrington and Ginty, 2013; Huang and Reichardt, 2001; Luo et al., 2007; Miller and Kaplan, 2001; Sharma et al., 2010). Retrograde NGF signals are propagated through the actions of signaling endosomes (Cosker and Segal, 2014; Howe and Mobley, 2005). NGF/TrkA complexes formed at distal axons are internalized and actively sorted into endosomes, a subset of which become mature, signaling-competent vesicles that undergo long-distance, microtubule mediated retrograde transport to the soma. By recruiting TrkA effectors, TrkA endosomes function as signaling platforms that convey trophic signals to support survival, maturation and synaptogenesis. Retrograde NGF/TrkA signaling endosomes are widely believed to be Rab5<sup>+</sup> early endosomes based on *ex vivo* biochemical and *in vitro* and *in vivo* immunocytochemical analyses, and because the membrane topology of TrkA receptors in early endosomes can, in principle, enable signal propagation (Cosker and Segal, 2014; Howe and Mobley, 2005).

The molecular identity of the TrkA signaling endosome remains controversial in part because of the technical difficulties of reliable and specific labeling of retrograde NGF-TrkA complexes. We have developed an *in vitro* compartmentalized chamber system in which neuronal cell bodies and distal axons are microfluidically isolated (Taylor et al., 2005). Further, the use of a *TrkA*<sup>FLAG</sup> knock-in mouse line generated in the lab, in which endogenous TrkA is tagged with a

FLAG epitope, enables visualization of trafficking of TrkA endosomes using anti-FLAG. This FLAG-TrkA transport assay that has proven to be a powerful technique for detecting and monitoring endogenous TrkA endosomes in neurons (Harrington et al., 2011; Sharma et al., 2010). Further, the combination of this assay and live cell imaging enables assessment of the dynamics of individual TrkA signaling endosomes and their associated regulators with extreme temporal and spatial precision (Lehigh et al., 2017). Another possible explanation for the controversy is that mechanisms of retrograde transport differ between neuronal types, so our experiments were performed using two major NGF-dependent neuronal cells: sympathetic neurons and TrkA<sup>+</sup> small-diameter sensory neurons. This strategy will allow for the first time a comparison of mechanisms of retrograde signaling in different neuronal populations. Taken together, our approach provides a unique opportunity to reveal the identity, mechanisms and functions of the NGF/TrkA signaling endosome in the nervous system.

## **Materials and Methods**

### **Mouse lines**

The *TrkA<sup>Flag</sup>* mouse line has been described (Sharma et al., 2010). Mice were handled and housed in accordance with Harvard Medical School and Johns Hopkins University IACUC guidelines.

### **Reagents and Antibodies**

Saponin was obtained from Sigma-Aldrich. The general caspase inhibitor, Boc-Asp(OMe)-FMK (BAF) was obtained from MP Biomedicals. The following antibodies were used: Rab5 (1:500), Rab7 (1:200, 1:1000 for immunoblot), Lamp1 (1:1000) (Abcam); P-TrkA Y490 and Y785 (1:2000, 1:100 for immuno-EM), P-PLC $\gamma$  (1:500, 1:50 for immuno-EM) (Cell Signaling); Flag (1 $\mu$ g/ml) (Sigma). Alexa Fluor secondary antibodies (Thermo Fisher Scientific) were used at 1:1000. Goat anti mouse or rabbit IgG F(ab')<sub>2</sub>-6nm/10nm gold secondary antibodies (Aurion)

were used at 1:50. Protein A-5nm/10nm gold antibodies were made by The Harvard Medical School EM Facility and used at a dilution of 1:50.

#### Molecular cloning, transfection and lentiviral infection

cDNAs of mouse Rab5, Rab7, CD63 and Hrs and human Rab7 were purchased from GE Healthcare. Individual cDNAs or fusion transgenes were cloned into the lentiviral vector FUW by In-Fusion cloning kit (Takara).

DNA plasmids were transfected into neuronal cells on DIV 3 using Lipofectamine 2000 (1 µg DNA: 4 µl Lipofectamine per well).

Lentivirus was generated, harvested and concentrated as previously described (Salmon and Trono, 2007). Neuronal cultures were infected on DIV 3 and experiments were performed 48~72 hr later.

#### Neuronal cell culture

Sympathetic neurons were cultured as described previously. Briefly, SCGs harvested from P0-P4 mice were dissociated and plated in mass cultures or compartmentalized microfluidic devices.

Neuronal cultures were maintained in DMEM supplemented with 10% FBS and NGF (50 ng/ml). Cytosine β-D-arabinofuranoside (AraC) was added from DIV 1-3 to eliminate proliferating fibroblast and glia cells. DRG sensory neurons were cultured as previously described.

#### The Flag-TrkA endosome transport assay

The Flag-TrkA assay was conducted as follows: an anti-Flag antibody was applied to the distal axon compartment (DA) of the microfluidic chamber and cells were incubated for 45 minutes at 4 °C. Axons were washed extensively to remove unbound antibody and NGF was applied to the

DA side. Cells were then incubated at 37 °C for indicated times to allow Flag-TrkA internalization, maturation and trafficking. To visualize internalized Flag-TrkA in distal axons, the DA compartment was washed with 0.5M NaCl/0.2M acetic acid to remove surface bound antibody prior to fixation.

#### Immunocytochemistry

To visualize proteins associated endosomes, a 0.025% saponin wash was performed first for 2 minutes. Cells were then fixed with 4% PFA for 10 minutes, blocked in 0.05% saponin and 2% serum, and subjected to antibody staining. TrkA<sup>+</sup> neurons in DRG cultures were identified by TrkA immunostaining and only those neurons were counted for quantification. Images were acquired by laser scanning confocal microscopy (Zeiss LSM 700).

#### Live cell imaging

To track Flag-TrkA endosome movement in real time, an anti-Flag antibody pre-conjugated to Alexa Fluoro secondary antibody was applied to the distal axon compartment during the 4 °C incubation step of the Flag-TrkA assay. Prior to imaging, culture medium was replaced by artificial cerebrospinal fluid (ACSF) that is phenol red free and CO<sub>2</sub> independent. Axons and cell bodies were imaged by spinning disk microscopy (Zeiss) at 37 °C in an environmental chamber at 2 frames per second (512×512 pixels) using a 63X oil immersion objective (1.40 NA). Time-lapse images were imported to ImageJ (NIH) and individual vesicles were tracked manually and analyzed by the MtrackJ plugin.

#### Electron microscopy

To detect retrogradely transported Flag-TrkA, transferrin, CTB or BSA, an anti-Flag antibody pre-conjugated to Protein A-5 nm gold secondary antibody, transferrin-gold (6 nm), CTB-gold (6



nm) or BSA-gold (6 nm) was applied to distal axons during the 4 °C incubation step of the Flag-TrkA assay, respectively. After the transport assay, cells were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer for 30 minutes at room temperature. Cells were then post-fixed with 1% osmium tetroxide, stained with 2% uranyl acetate and 2% tannic acid to enhance membrane contrast, dehydrated in series of ethanol followed by embedding in EPON resin. The next day, coverslips were removed and areas containing cells were randomly selected and mounted. Ultrathin sections (70 nm) were collected, stained with lead citrate, and were examined on a JOEL electron microscope.

#### Immunoelectron microscopy

Flag-TrkA assay was performed as above. Cells were fixed in 4% PFA and 0.15% glutaraldehyde for 30 minutes. Cells were then washed with 0.1% sodium borohydride for 30 minutes at room temperature to quench excessive free aldehyde and blocked in PBS with 10% serum, 0.05% saponin and 0.5% gelatin for 2 hours. Cells were incubated in primary antibodies in blocking solution overnight. After extensive washing, cells were incubated with 10 nm gold secondary antibodies overnight and washed again. Cells were then processed for EM.

The Flag-TrkA live imaging assay was developed and optimized by Dr. Kathryn Lehigh. I am grateful for her contributions and technical advices.

## Results

We investigated the vesicular nature, molecular composition, maturation, and signaling dynamics of TrkA<sup>+</sup> signaling endosomes using a *TrkA<sup>Flag</sup>* knockin mouse line, which expresses Flag epitope-tagged TrkA from the endogenous TrkA locus, and an *in vitro* compartmentalized microfluidic sympathetic neuron culture system that allows us to monitor internalization, sorting and retrograde trafficking of Flag-TrkA<sup>+</sup> endosomes (Figure 2.1A). We first sought to define the

ultrastructural features of retrogradely transported TrkA<sup>+</sup> endosomes. To accomplish this, compartmentalized *TrkA<sup>Flag</sup>* sympathetic neurons and an anti-Flag antibody pre-conjugated to Protein A-5nm gold were used to visualize retrogradely transported Flag-TrkA<sup>+</sup> endosomes by electron microscopy (EM). While application of neither the primary antibody, nor the gold secondary antibody alone to distal axons of compartmentalized *TrkA<sup>Flag</sup>* neurons yielded electron-dense structures detectable by EM, application of anti-Flag antibody that was pre-conjugated to Protein A-5nm gold to *TrkA<sup>Flag</sup>* neurons, but not wild-type neurons, labeled electron dense structures in axons that were readily apparent by EM (Figure 2.1E). As expected, newly internalized, gold-labeled TrkA receptors in distal axons were found in single-membrane vesicular structures in close proximity to the plasma membrane, which is a defining feature of early endosomes (Figure 2.1B). Surprisingly, and in stark contrast, one hour after NGF application to distal axons, retrogradely transported TrkA receptors in proximal axons and cell bodies were found mainly in MVBs ( $87.9\% \pm 5.0\%$ ) and, to a much lesser extent, single-membrane vesicles (SVs,  $9.5\% \pm 2.8\%$ ) or lysosomes ( $2.6\% \pm 1.3\%$ ; Figure 2.1C, D). The gold-labeled Flag-TrkA receptors were localized both to the limiting membrane and intraluminal vesicles (ILVs) of MVBs (Figure 2.1C). Therefore, following NGF treatment of distal axons, newly internalized TrkA in distal axons is associated with early endosomes, whereas following retrograde transport to proximal axons and cell bodies TrkA is predominantly associated with MVBs.

To complement the EM analyses and to define molecular features of TrkA signaling endosomes in sympathetic neuron axons, we next used the Flag-TrkA transport assay to visualize TrkA<sup>+</sup> endosomes and determine the extent to which they are associated markers of distinct types of vesicular compartments (Figure 2.2). Consistent with the EM observations, following application

of NGF exclusively to distal axons, newly internalized TrkA receptors in distal axons were mainly associated with Rab5-labeled early endosomes (Figure 2.2A, top). However, the fraction of total internalized Flag-TrkA colocalized with Rab5 in distal axons decreased during the next hour, coincident with an increase of Flag-TrkA colocalized with Rab7, a small GTPase that regulates the functions of MVBs and late endosomes (LEs) (Figure 2.2B). Strikingly, Flag-TrkA punctae within proximal axons, which represent mature, retrogradely transported endosomes, were mainly associated with MVB/LE markers, including Rab7, Hrs and CD63 and, to a much lesser extent, with markers of early endosomes (Rab5), recycling endosomes (Rab11) and lysosomes (Lamp1) (Figure 2.2A, B, D, E).

We also directly visualize movement of actively transported TrkA endosomes in real time by imaging *TrkA<sup>Flag</sup>* neurons expressing fluorophore-tagged endosomal proteins using a modified Flag-TrkA assay. In this live cell imaging paradigm, anti-Flag antibody pre-conjugated with fluorescent secondary antibody was applied to distal axons of *TrkA<sup>Flag</sup>* neurons. This live cell Flag-TrkA imaging assay enabled specific visualization of Flag-TrkA endosomes because the fluorescent signal was undetectable in wild type neurons (data not shown). We expressed in *TrkA<sup>Flag</sup>* neurons EGFP-tagged Rab5 or Rab7 using a lentiviral delivery system. These constructs did not compromise NGF-dependent survival of sympathetic neurons (Figure 2.3A). Live cell imaging of EGFP-Rab5-expressing *TrkA<sup>Flag</sup>* neurons revealed that the majority of EGFP-Rab5<sup>+</sup>/Flag-TrkA endosomes in distal axons are stationary or move bi-directionally for a short distance (Figure 2.3B, yellow arrow heads); very few of the EGFP-Rab5<sup>+</sup>/Flag-TrkA<sup>+</sup> punctae were co-transported retrogradely into proximal axons of the cell body compartment (Figure 2.3C-E). In contrast, the majority of retrogradely transported Flag-TrkA<sup>+</sup> endosomes observed in proximal axons were associated with EGFP-Rab7 (Figure 2.3C, white arrow heads).

Furthermore, unlike EGFP-Rab5<sup>+</sup>/Flag-TrkA<sup>+</sup> endosomes, most of the EGFP-Rab7<sup>+</sup> TrkA endosomes exhibited consistent and processive movement in the retrograde direction; very few were stationary or moved bi-directionally (Figure 2.3C-E; Movie S2). The percentage of Flag-TrkA punctae co-transported with EGFP-Rab5 or EGFP-Rab7 were similar to the percentages of colocalization observed in the fixed cell immunolabeling experiments. Similar observations were found using a different MVB marker, CD63 (Figure 2.3F). These observations, taken together, demonstrate that retrograde Flag-TrkA<sup>+</sup> endosomes are associated with markers of MVBs or LEs, and not early endosomes.

In the canonical ligand-receptor endocytic pathway, after receptor tyrosine kinase (RTK) activation and internalization, ligand-receptor complexes follow an endocytic route from early endosomes to MVBs and finally to LEs and lysosomes (Bergeron et al., 2016). The prevailing view is that signals from activated RTKs emanate primarily from the inner leaflet of the plasma membrane, and then, following internalization, from early endosomes (Cosker et al., 2008; Delcroix et al., 2003; Harrington and Ginty, 2013; Sorkin and von Zastrow, 2009; Zoncu et al., 2009). In this model, sorting of RTKs into MVBs is mainly considered in the context of signal deactivation and ligand/receptor degradation. Alternatively, MVBs may function to promote signaling by mediating sorting and degradation of negative regulators (Taelman et al., 2010). To ask whether retrogradely transported TrkA<sup>+</sup> MVBs have signaling capacity, we monitored their association with effectors of NGF/TrkA signaling. NGF-induced TrkA dimerization and autophosphorylation, particularly on tyrosine residues 490 and 785, signifies receptor activation and functions to trigger downstream signaling cascades, including the Ras/ERK, PLC $\gamma$ , and PI3K pathways, by recruiting signaling pathway effector proteins to the membrane (Figure 2.4A) (Huang and Reichardt, 2001; Sofroniew et al., 2001; Watson et al., 2001). Indeed, both TrkA<sup>+</sup>

early endosomes and MVBs within axons contain P-TrkA Y490 and Y785, indicative of an active TrkA signaling state (Figure 2.4B, C). Moreover, most TrkA<sup>+</sup> MVBs in axons and cell bodies were observed in association with the active form of a key TrkA effector, PLC $\gamma$  phosphorylated on tyrosine residue 783, suggesting an ability of TrkA MVBs to recruit and activate core TrkA signaling pathway effectors (Figure 2.4 B-D).

To identify membrane compartments in which retrograde TrkA receptors are capable of signaling, we performed immuno-gold labeling of neurons using the Flag-TrkA retrograde transport assay, in conjunction with antibodies against both P-TrkA and P-PLC $\gamma$ . Many P-TrkA and P-PLC $\gamma$  labeled structures in cell bodies were observed to be associated with membranes, and the extent of labeling was greatly diminished following treatment of K252a, a Trk kinase inhibitor, thus confirming the specificity of the immuno-gold labeling strategy (Figure 2.5A). When NGF was applied to cell bodies, co-localization of Flag-TrkA with P-TrkA or P-PLC $\gamma$  was observed at the plasma membrane as well as associated with early endosomes (data not shown). In contrast, when NGF was applied to distal axons, both P-Trk and P-PLC $\gamma$  were observed co-localized with retrogradely transported Flag-TrkA on the limiting membrane and intraluminal vesicles (ILVs) of MVBs in cell bodies in a K252a-dependent manner (Figure 2.5B, C), which is consistent with findings of the immunolabeling experiments. We also assessed the extent of co-localization between TrkA<sup>+</sup> MVBs and the lysosomal marker Lamp1 in axons and cell bodies. While over 50% of CD63 punctae in both distal and proximal axons were Lamp1<sup>+</sup>, very few CD63/Flag-TrkA double-labeled punctae were associated with lysosomes in axons and cell bodies ( $8.5 \pm 2.2\%$ ; Figure 2.4D). Taken together, both TrkA<sup>+</sup> early endosomes and MVBs recruit NGF/TrkA signaling effector proteins, suggesting that both are capable of TrkA effector signaling, and retrograde TrkA<sup>+</sup> MVBs are uniquely not degradative.

## Conclusions

In summary, by using ultrastructural, immunocytochemical and live imaging analyses, I showed that Rab7<sup>+</sup> MVBs, not Rab5<sup>+</sup> early endosomes, are major carriers of retrograde NGF signals in compartmentalized sympathetic neuronal cultures. Moreover, retrograde TrkA MVBs associate key effectors of NGF-TrkA signaling pathways, indicating they are bona fide signaling endosomes. These findings expand the text-book view of MVB function being solely degradative. Rather, MVBs can undergo long-range axonal movement and transmit essential survival signals in developing neurons.

**Figure 2.1. Retrograde TrkA<sup>+</sup> endosomes are predominantly of multi-vesicular, not single-vesicular, ultrastructure.**

(A) Schematic of the Flag-TrkA endosome transport assay. DA: distal axons. PA: proximal axons.

(B) Newly internalized Flag-TrkA is sorted into early endosomes in distal axons. The Flag-TrkA assay was performed in compartmentalized sympathetic neurons using pre-conjugated anti-Flag antibody with Protein A-5 nm gold. Cells were fixed 5 mins post NGF application and processed for EM. White arrows denote Flag-TrkA gold particles within an endosome. n=3. Scale bar: 100 nm.

(C, D) The Flag-TrkA transport assay was performed in compartmentalized sympathetic neurons using pre-conjugated anti-Flag antibody with Protein A-5 nm gold. Cells were fixed 1hr post NGF application and processed for EM. The percentage of Flag-TrkA gold particles localized to MVBs, single-membrane vesicles (SVs) or lysosomes was quantified (D). Note the presence of the Flag epitope on both the membrane of the intraluminal vesicles (white arrows) and the limiting membrane of the MVB (yellow arrows). High magnification images of the boxed areas are shown in the bottom panel.

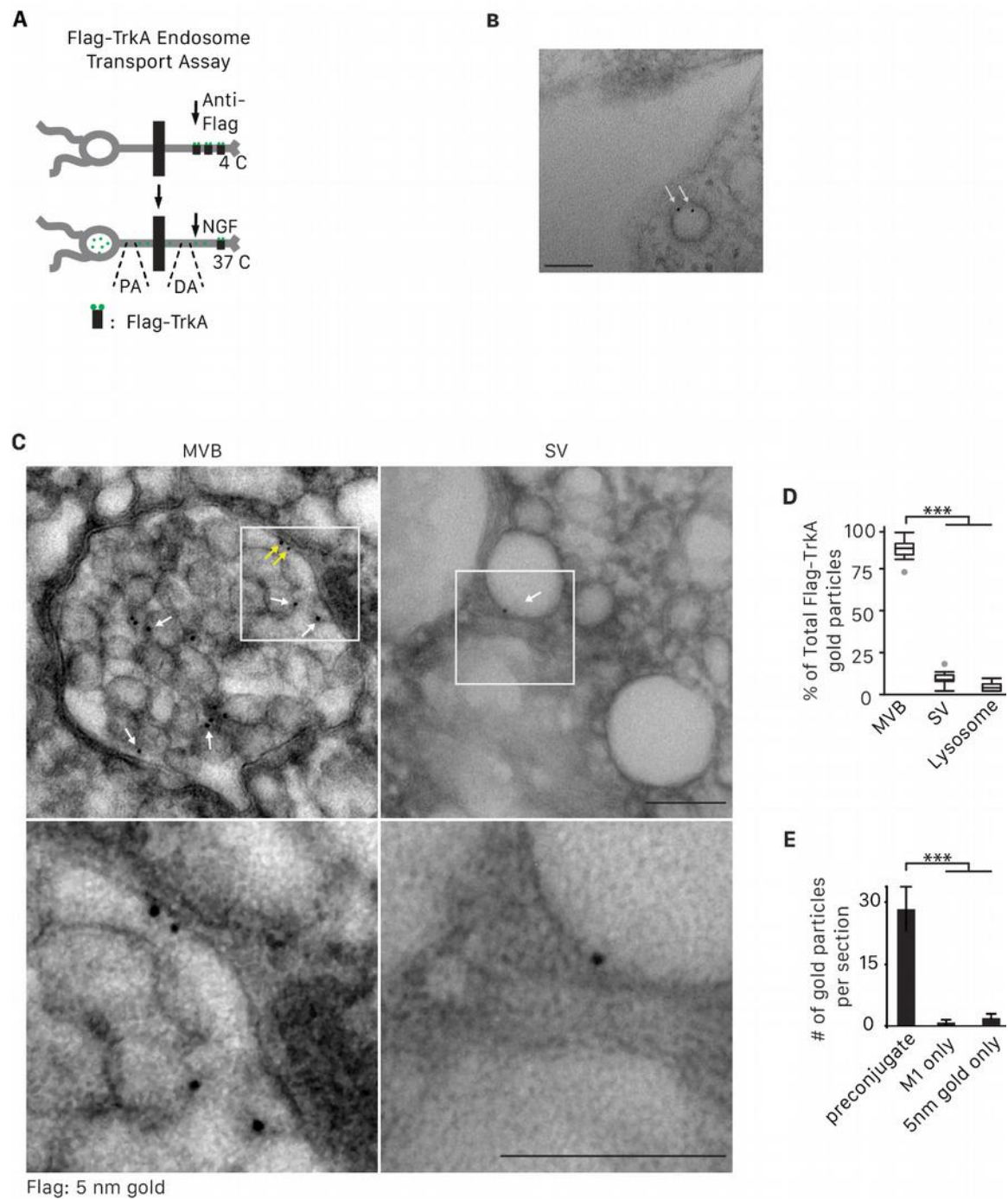
(E) The Flag-TrkA assay was performed using pre-conjugated primary and secondary antibodies, or primary or secondary antibody only. Cells were fixed 1 hr post NGF stimulation and the number of gold particles per EM section was counted (n = 4). Scale bar: 100 nm.

Data are presented in box plot (C) or represented as mean  $\pm$  standard error of the mean (SEM)

(E). In box plots, the top and the bottom of the central rectangle represents the 75<sup>th</sup> and 25<sup>th</sup> percentile value, respectively, and the line inside represents the median; the whisker on either

side extends to the data point that is within the range of variation ( $1.5 \times (75^{\text{th}} \text{ percentile} - 25^{\text{th}} \text{ percentile})$ ) and data points beyond that range are plotted as individual dots. \*\*\* $p < 0.001$  by one-way ANOVA followed by Tukey's *post-hoc* correction.





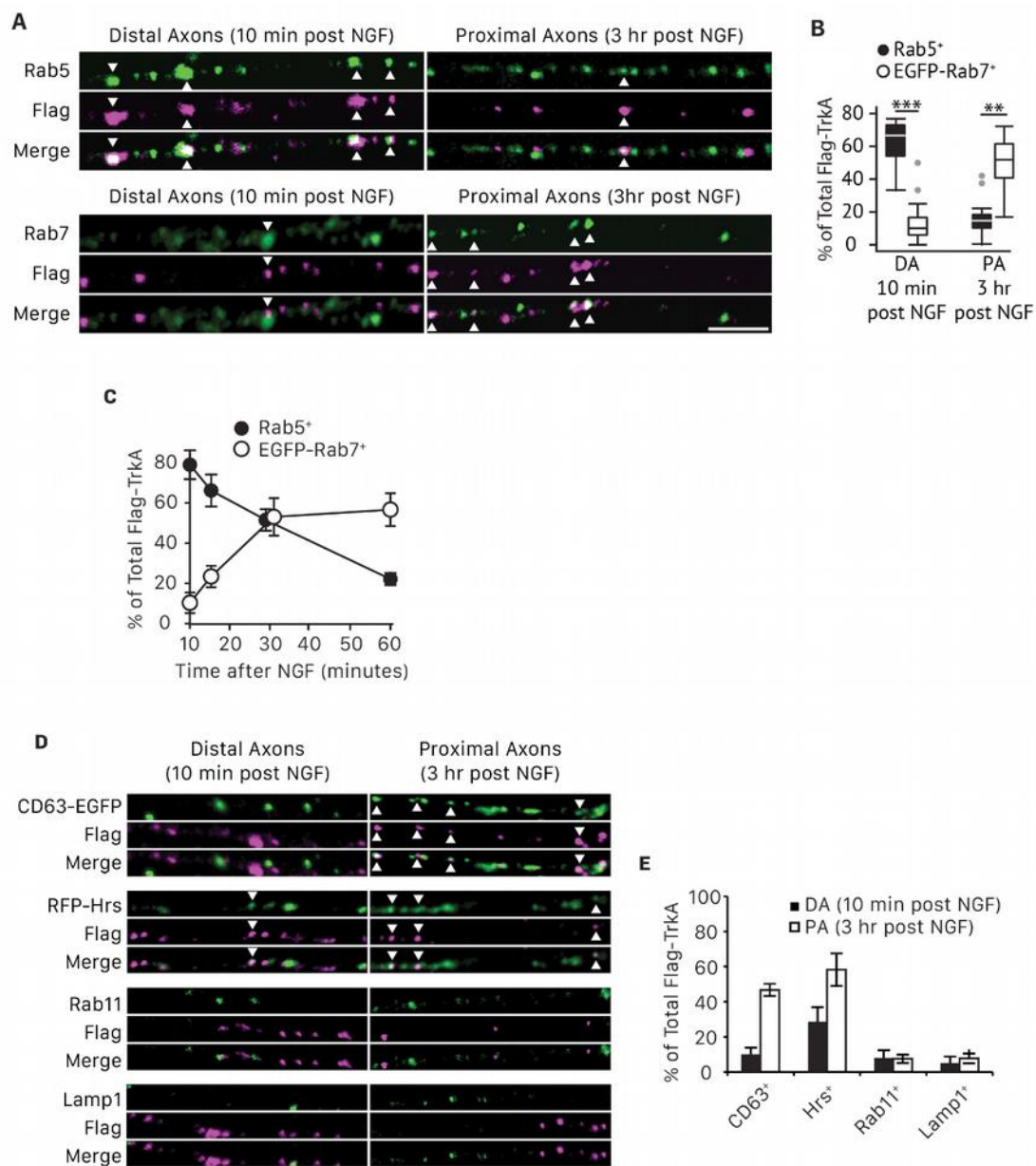
**Figure 2.2. Multivesicular bodies, not early endosomes, are major carriers of retrograde TrkA signals in sympathetic neurons.**

(A, B) The Flag-TrkA endosome transport assay was performed in sympathetic neurons grown in compartmentalized microfluidic culture and infected with a lentivirus expressing EGFP-Rab7. The percentage of Flag-TrkA punctae (magenta) colocalized with the MVB marker EGFP-Rab7 or the EE marker Rab5 (green) in distal axons (DA) 10 minutes post NGF application or in axons proximal to cell bodies (PA) 3 hr post NGF application was quantified ( $n = 5$ ). For DA experiments, cells were washed with NaCl/acetic acid to remove surface bound Flag signal prior to fixation. Scale bar: 5  $\mu\text{m}$ .

(C) Colocalization of Flag-TrkA endosomes with EGFP-Rab5 or EGFP-Rab7 in distal axons post internalization over time was quantified ( $n = 3$ ).

(D, E) Colocalization between Flag-TrkA (magenta) and CD63-EGFP, RFP-Hrs, Rab11 and Lamp1 (green) in distal and proximal axons of compartmentalized sympathetic neurons. For distal axon experiments, cells were fixed 10 mins post NGF application and were stripped with NaCl/acetic acid to remove surface Flag antibody. For proximal axon experiments, cells were incubated for 3 hr post NGF application ( $n = 3$ ). Scale bar: 10  $\mu\text{m}$ .

Data are presented in box plot (B) or represented as mean  $\pm$  SEM (C, E). \*\* $p < 0.01$  and \*\*\* $p < 0.001$  by two tailed unpaired Student's  $t$  test.



**Figure 2.3. Retrogradely transported TrkA is co-transported with MVB, but not early endosome proteins.**

(A) Compartmentalized sympathetic neurons were infected with a virus expressing an empty vector, EGFP-Rab5 or EGFP-Rab7, and neuronal survival was assessed. Expression of EGFP-Rab5 or EGFP-Rab7 did not affect retrograde NGF-dependent survival of sympathetic neurons (n = 3).

(B-D) Sympathetic neurons grown in compartmentalized culture were infected with lentivirus expressing EGFP-Rab7 or EGFP-Rab5. The Flag-TrkA assay was performed using pre-conjugated anti-Flag antibody and Alexa Fluoro secondary antibody during the 4°C incubation step. Flag-TrkA (magenta) and EGFP (green) were then imaged consecutively in axons.

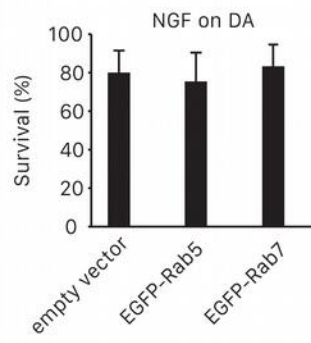
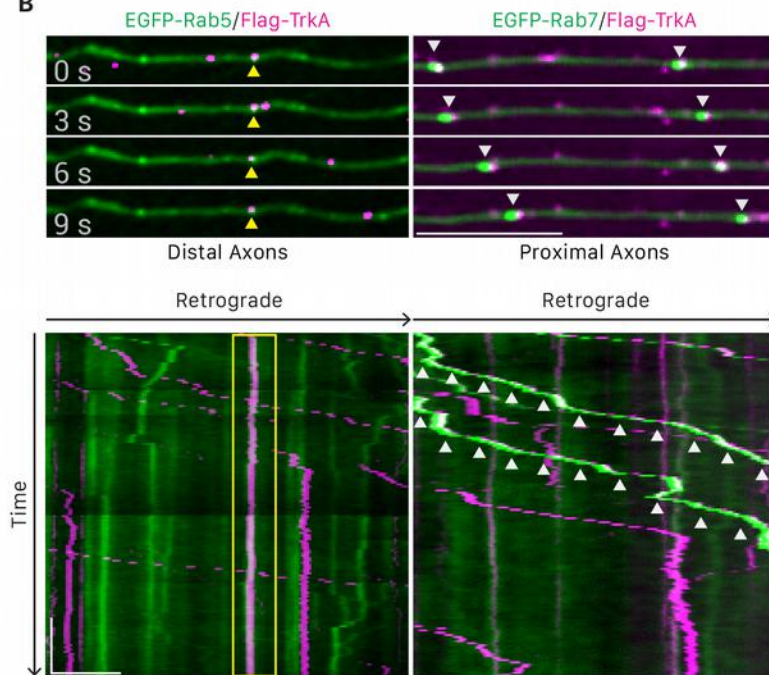
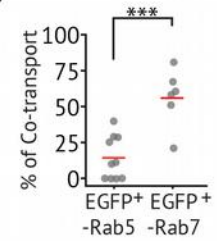
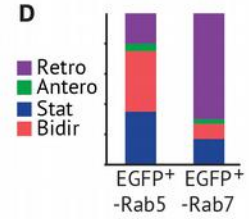
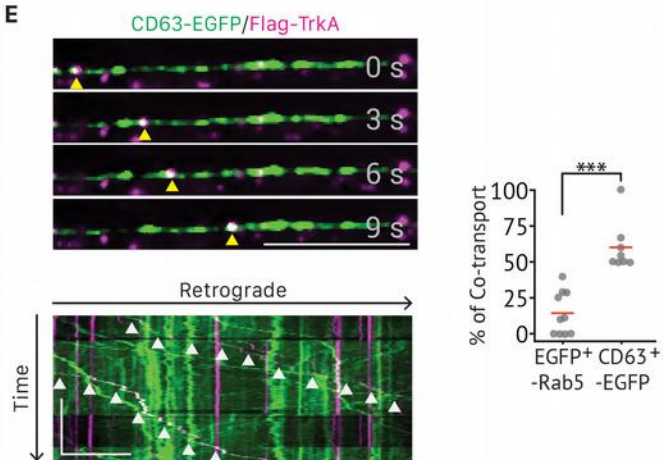
Representative time-lapse images of each type of TrkA endosomes are shown (top panel).

Kymographs of time-lapse images are shown in the bottom panel. Arrowheads denote individual endosomes. Scale bar: 10 µm; 1 min. The percentage of Flag-TrkA punctae co-transported with either marker was quantified (C). The directionality of retrograde TrkA MVBs and EEs are shown in (D). A total of 168 (Rab5) and 207 (Rab7) endosomes were scored in 4 independent experiments for each condition.

(E) Sympathetic neurons grown in compartmentalized culture were infected with lentivirus expressing CD63-EGFP. The Flag-TrkA assay was performed using pre-conjugated anti-Flag antibody and Alexa Fluoro secondary antibody during the 4°C incubation step. Flag-TrkA (magenta) and EGFP (green) were then imaged consecutively in axons. Representative time-lapse images of each type of TrkA endosomes are shown (top panel). The kymograph of time-lapse images are shown in the bottom panel. Arrowheads denote individual endosomes. Scale bar: 10 µm; 1 min. The percentage of Flag-TrkA punctae co-transported with CD63-EGFP was

quantified. A total of 173 endosomes were scored in 4 independent experiments for each condition.

Data are represented as mean  $\pm$  SEM (A) or presented in dot plots (C, E). In dot plots, individual data points (dots) and the mean (red line) are shown. \*\*\* $p < 0.001$  by two tailed unpaired Student's  $t$  test.

**A****B****C****D****E**

**Figure 2.4. Retrograde TrkA<sup>+</sup> MVBs associate with key effectors of the NGF/TrkA signaling pathway.**

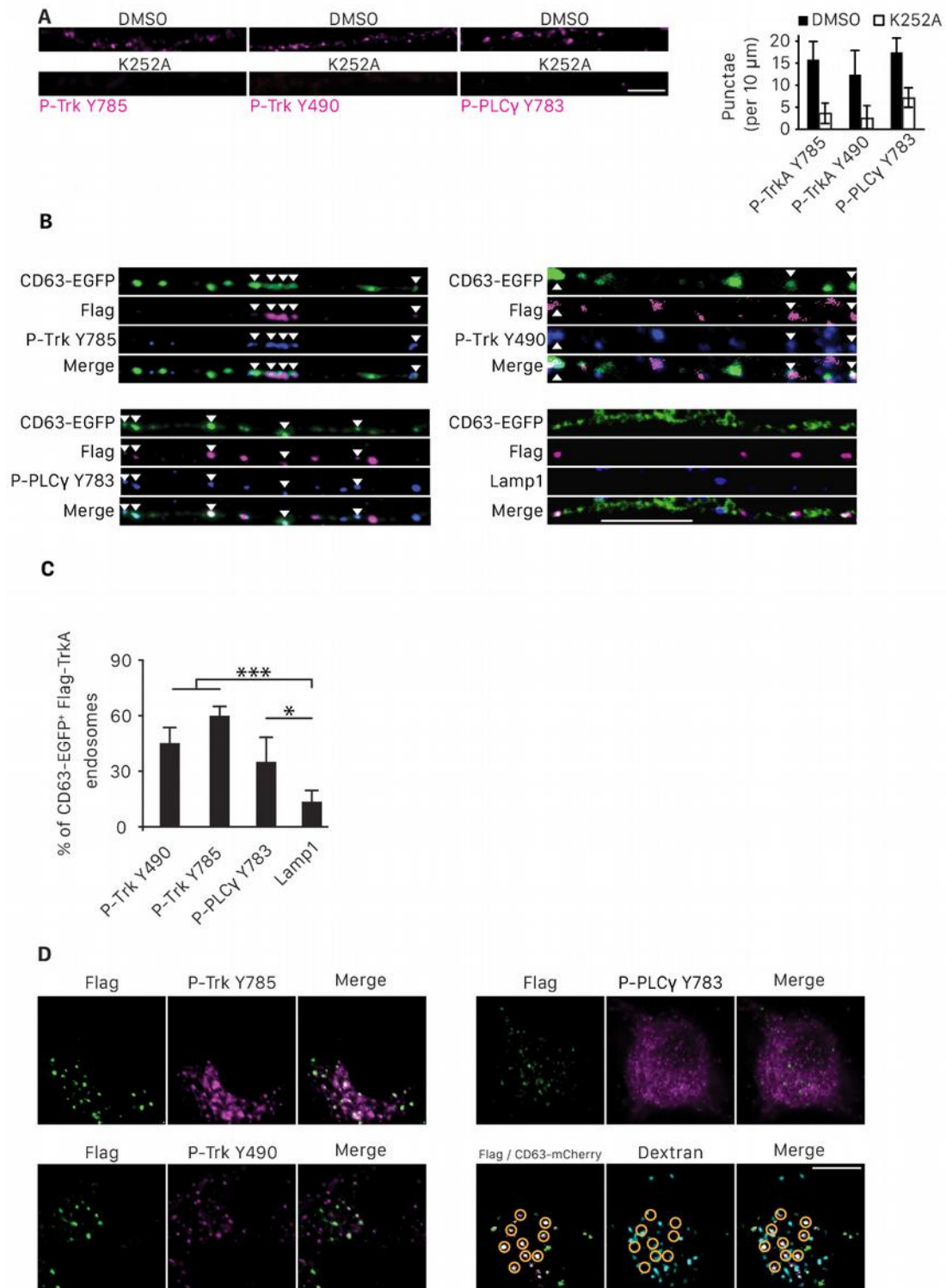
(A) Compartmentalized sympathetic neurons were NGF- and serum-deprived for 6 hr and then stimulated with NGF in the presence of either DMSO or K252a for 20 minutes in distal axons. Cells were then immunostained for P-Trk Y490, P-Trk Y785 and P-PLC $\gamma$  (n = 3).

(B, C) The Flag-TrkA endosome transport assay was performed in compartmentalized sympathetic neurons expressing CD63-EGFP. The extent of CD63-EGFP<sup>+</sup> Flag-TrkA (green/magenta) punctae in proximal axons colocalized with P-Trk Y490 and Y785, P-Plc $\gamma$  Y783 and Lamp1 (blue) was quantified (C) (n = 3). Scale bar: 10  $\mu$ m.

(D) The Flag-TrkA assay was performed in compartmentalized sympathetic neurons. Cells were fixed 3 hr post NGF application and colocalization between Flag-TrkA (green) with P-Trk Y490, P-Trk Y785, P-PLC $\gamma$  Y783 (Magenta) or lysosomes (blue) in cell bodies was assessed (n = 3). Lysosomes were labeled by pre-loading the cells with Dextran (blue) for 2 hr prior to the Flag assay. Yellow circles denote CD63-mCherry<sup>+</sup> Flag-TrkA endosomes. Scale bar: 10  $\mu$ m.

Data are represented as mean  $\pm$  SEM. \*p < 0.05 and \*\*p < 0.01 by one-way ANOVA followed by Tukey's *post-hoc* correction.





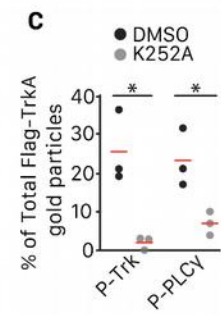
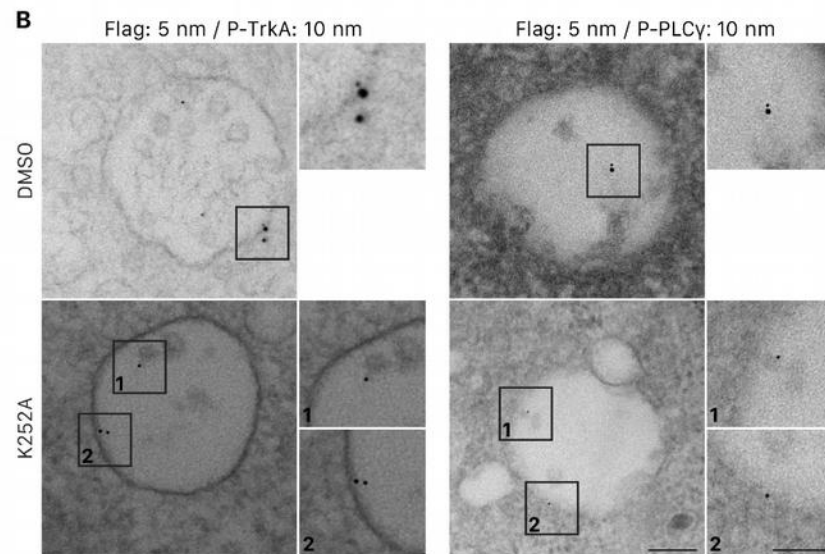
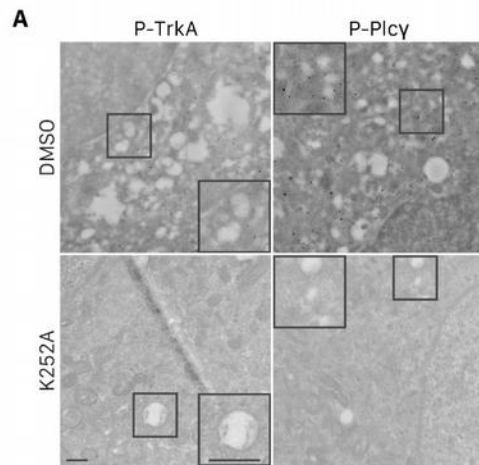


**Figure 2.5. Retrogradely transported TrkA in MVBs associate with key effectors of the NGF/TrkA signaling pathway.**

(A) Sympathetic neurons grown in mass culture were NGF-deprived for 16 hr and were then stimulated with NGF for 1 hr in the presence of DMSO (vehicle control) or K252a (200 nM), a Trk kinase inhibitor. P-TrkA and P-Plc $\gamma$  signals in cell bodies were assessed by pre-embed immunogold labeling (n = 3). Insets: high magnification images of the boxed areas.

(B, C) The Flag-TrkA assay was performed in compartmentalized sympathetic neurons using pre-conjugated anti-Flag antibody with a 5 nm gold secondary antibody in the presence of DMSO or K252a in the cell body compartment. Neurons were fixed 1 hr post NGF stimulation and P-TrkA and P-Plc $\gamma$  signals were revealed by immunogold labeling (10 nm). The extent of Flag-TrkA gold particles associated with P-TrkA or P-PLC $\gamma$  in cell bodies was assessed (n = 3). Scale bar: 100 nm.

Data are presented in dot plot. \*p < 0.05 by one-way ANOVA followed by Tukey's *post-hoc* correction.



**Chapter 3: Rab7 mediates TrkA transport and signaling *in vivo* and  
*in vitro***

## Introduction

Target fields support retrograde signaling, survival, and maturation of neurons through signaling endosomes (Harrington and Ginty, 2013; Howe and Mobley, 2005). In Chapter 2, I showed that ultrastructurally- and molecularly-defined MVBs are major carriers of retrograde NGF signals. However, the molecular mechanisms that underlie the formation, maturation, and signaling capacity of retrograde TrkA MVBs are unclear. A key class of proteins that define endosomal membrane domains and regulate endosome function and maturation is the Rab small GTPase (Zerial and McBride, 2001). Rab proteins often associate and mark distinct types of endosomal structures. For example, Rab5 labels early endosomes; Rab 7 labels MVBs and late endosomes; and Rab11 and 14 label recycling endosomes (Zerial and McBride, 2001). Rab proteins play critical roles in various aspects of formation, cargo sorting, movement and turnover of endosomes (Zerial and McBride, 2001). A Rab5-to-Rab7 switch has been implicated in maturation of early endosomes to late endosomes (Poteryaev et al., 2010; Rink et al., 2005). Rab7 also regulate positioning, movement and lysosomal fusion of late endosomes (Johansson et al., 2007; Rocha et al., 2009). Most of what we know about Rab protein functions derived from studies in non-neuronal cells, and thus it is important to re-evaluate their roles and functional requirement in endosomal dynamics in neurons and in the context of retrograde transport and signaling.

## Materials and Methods

### Mouse lines

The *TrkA<sup>Flag</sup>*, *TH<sup>2a-CreER</sup>*, and *Rab7<sup>fllox</sup>* mouse lines have been described (Abraira et al., 2017; Roy et al., 2013; Sharma et al., 2010). Genotyping was performed using the following primer sets:

*Rab7<sup>fllox</sup>*: CTCACTCACTCCTAAATGG and TTAGGCTGTATGTATGTGC; *TH<sup>2a-CreER</sup>*:

CATGCCCATATCCAATCTCC and CTGGAGCGCATGCAGTAGTA. Mice were handled and housed in accordance with Harvard Medical School and Johns Hopkins University IACUC guidelines.

### **Reagents and Antibodies**

K252a, 1NMPP1 and nocodazole were obtained from EMD Millipore. Saponin and 3,3'-Diaminobenzidine (DAB) was obtained from Sigma-Aldrich. The general caspase inhibitor, Boc-Asp(OMe)-FMK (BAF) was obtained from MP Biomedicals. 6 nm gold-conjugated transferrin, CTB or BSA were obtained from Electron Microscopy Sciences. The following antibodies were used: NeuN (1:1000), Rab5 (1:500), Rab7 (1:200, 1:1000 for immunoblot), Lamp1 (1:1000) (Abcam); P-TrkA Y490 and Y785 (1:2000, 1:100 for immuno-EM), P-PLC $\gamma$  (1:500, 1:50 for immuno-EM) (Cell Signaling); VACHT (1:1000, Enzo Life Sciences); Homer1 (1:1000, Synaptic Systems); Flag (1 $\mu$ g/ml) (Sigma). Alexa Fluor secondary antibodies (Thermo Fisher Scientific) were used at 1:1000. Goat anti mouse or rabbit IgG F(ab')<sub>2</sub>-6nm/10nm gold secondary antibodies (Aurion) were used at 1:50. Protein A-5nm/10nm gold antibodies were made by The Harvard Medical School EM Facility and used at a dilution of 1:50.

### **Molecular cloning, transfection and lentiviral infection**

cDNAs of mouse Rab5, Rab7, CD63 and Hrs and human Rab7 were purchased from GE Healthcare. Individual cDNAs or fusion transgenes were cloned into the lentiviral vector FUW by In-Fusion cloning kit (Takara). Sequences of 21mer siRNAs against mouse Rab7 and RILP were obtained from TRC library (Moffat et al., 2006). shRNA oligos were synthesized, annealed and cloned into the lentiviral pLLX vector as described (Zhou et al., 2006). Five shRNA clones were pooled for lentivirus preparation (GAAGTTCAGTAACCAGTACAA; GCGGCAGTATTCTGTACAGTA; GCCCTTAAACAGGAAACAGAA;

TGAACCCATCAAACCTGGACAA; TGCTGTGTTCTGGTGTGTTGAT). A scrambled shRNA was used as control (CCTAAGGTAAAGTCGCCCTCG).

DNA plasmids were transfected into neuronal cells on DIV 3 using Lipofectamine 2000 (1 µg DNA: 4 µl Lipofectamine per well).

Lentivirus was generated, harvested and concentrated as previously described (Salmon and Trono, 2007). Neuronal cultures were infected on DIV 3 and experiments were performed 48~72 hr later.

## Results

We previously found that Rab7, a small GTPase that regulates functions of MVBs and Les (Lebrand et al., 2002; Vanlandingham and Ceresa, 2009), is associated with immunopurified TrkA<sup>+</sup> endosomes (Harrington et al., 2011). Moreover, Rab7 is a major regulator of vesicular trafficking (Wandinger-Ness and Zerial, 2014). In motor neurons, retrograde transport of a modified tetanus toxin is mediated by Rab7<sup>+</sup> endosomes and is dependent on Rab7's GTPase activity (Deinhardt et al., 2006). Therefore, we asked whether Rab7 mediates long-range retrograde TrkA trafficking and signaling in sympathetic neurons using a combination of *in vivo* and *in vitro* analyses. Mice harboring a *TH*<sup>-2a-CreER</sup> knock-in allele (Abraira et al., 2017) and a conditional allele of *Rab7* (Roy et al., 2013) were generated to examine the role of Rab7 in sympathetic neurons *in vivo* (Figure 3.1). To ask whether Rab7 plays a role in NGF-dependent survival signaling in sympathetic neurons during early stages of development, *TH*<sup>-2a-CreER</sup>; *Rab7*<sup>fl/fl</sup> mice were treated with tamoxifen at E14, resulting in a partial loss of Rab7 protein during the critical period of NGF-dependent sympathetic neuron survival (Figure 3.1A). Superior cervical ganglia (SCGs) were harvested at P7 and the number of sympathetic neurons was counted. Compared to *TH*<sup>-2a-CreER</sup>; *Rab*<sup>+/+</sup> littermate controls, *TH*<sup>-2a-CreER</sup>; *Rab7*<sup>fl/fl</sup> animals exhibited ~60%

neuronal cell loss (Figure 3.1 C, D). To address the role of Rab7 in TrkA signaling in sympathetic neuronal soma and dendrites postnatally, *TH*<sup>2a-CreER</sup>; *Rab7*<sup>ff</sup> mice were treated with tamoxifen at P7 and P8 and SCGs were harvested at P14, a time following the period of NGF-dependent survival of sympathetic neurons. TrkA signaling in sympathetic neurons was assessed using antibodies that specifically recognize the phosphorylated TrkA tyrosine residues 490 or 785; these phosphorylation events are indicative of the active form of TrkA. Interestingly, a marked reduction of P-TrkA punctae was observed in sympathetic ganglia of *TH*<sup>2a-CreER</sup>; *Rab7*<sup>ff</sup> mice compared to *TH*<sup>2a-CreER</sup>; *Rab7*<sup>ff/+</sup> controls (Figure 3.1E, F). Moreover, in the absence of Rab7, a decrease in both pre- and post-synaptic specializations visualized using antibodies against the vesicular acetylcholine transporter (VAChT) and Homer1, respectively, was observed (Figure 3.1E, F). Therefore, Rab7 functions in sympathetic neurons *in vivo* to support NGF-dependent survival and synapse formation.

To investigate the role of Rab7 in retrograde survival signaling in sympathetic neurons under conditions in which the time of application of NGF exclusively to distal axons is controlled, we undertook an *in vitro* approach using compartmentalized, microfluidic chambers that separate distal axons (DA) from cell bodies and proximal axons (PA) (Harrington et al., 2011; Sharma et al., 2010). Rab7 was ablated in *Rab7*<sup>ff</sup> sympathetic neurons grown in compartmentalized chambers by infecting them with a lentivirus expressing Cre recombinase, or in wild-type compartmentalized sympathetic neurons infected with a lentivirus expressing an shRNA against Rab7, which greatly reduced Rab7 protein levels (Figure 3.1B). NGF was then applied exclusively to distal axons to evaluate the role of Rab7 in retrograde TrkA signaling. Consistent with our *in vivo* analysis, retrograde TrkA signaling (Figure 3.1G, H) and survival (Figure 3.1I, J) were compromised in neurons lacking Rab7 *in vitro*. This was not due to a non-

specific or global effect on neuronal survival, since addition of NGF directly to cell bodies and proximal axons of neurons lacking Rab7 supported their survival (Figure 3.1I, J). To ask whether Rab7 is necessary for retrograde TrkA transport, we employed a previously-described Flag-TrkA transport assay (Sharma et al., 2010). This assay combines the use of a *TrkA<sup>Flag</sup>* knockin mouse line, which expresses Flag epitope-tagged TrkA from the endogenous TrkA locus, and the *in vitro* compartmentalized microfluidic culture system, to monitor internalization, sorting and retrograde trafficking of Flag-TrkA<sup>+</sup> endosomes in *TrkA<sup>Flag</sup>* neurons, but not wild-type neurons. Using this assay, we found that expression of the Rab7 shRNA in sympathetic neurons virtually abolished retrograde transport of Flag-TrkA<sup>+</sup> endosomes (Figure 3.2A, B). On the other hand, Rab7 depletion did not affect TrkA internalization in distal axons (Figure 3.2A, DA). To assess the specificity of the Rab7 shRNA treatment, we used two Rab7 replacement constructs, one encoded by a human Rab7 cDNA that is resistant to the shRNA and another encoded by a mouse Rab7 cDNA that is the target of the shRNA. Expression of hRab7, but not mRab7, in sympathetic neurons treated with the Rab7 shRNA restored both retrograde TrkA transport and survival (Figure 3.2 A, B). Similar observations were found using TrkA<sup>+</sup> DRG sensory neurons, suggesting that Rab7 is a general mediator of retrograde TrkA transport and survival across different neuronal types (Figure 3.2 C, D). Taken together, these findings indicate that Rab7 mediates retrograde TrkA transport and signaling both *in vivo* and *in vitro*.

Rab7 has been implicated in early-to-late endosome transition, functions and maturation of late endosomes (Lebrand et al., 2002; Rink et al., 2005; Vanlandingham and Ceresa, 2009). To ask where Rab7 might function in the context of TrkA endosome maturation, we tested the necessity of Rab7 for formation of TrkA EEs and MVBs in distal axons of sympathetic neurons. Interestingly, comparable levels of Rab5<sup>+</sup> or CD63-mCherry<sup>+</sup> Flag-TrkA endosomes were found



in neurons expressing control or Rab7 shRNA, suggesting that Rab7 is not required for formation of TrkA EEs and MVBs in (Figure 3.3A-C).

Next, we sought to ask whether Rab7 is required for TrkA MVBs to become transport competent. We focused on one Rab7 effector protein, RILP, that has been shown to mediate late endosome positioning and dynein-dependent movement along microtubules (Johansson et al., 2007; Jordens et al., 2001). We found that RILP-EGFP was co-localized and co-transported with retrograde Flag-TrkA endosomes in proximal axons of compartmentalized sympathetic neurons (Figure 3.3D-G). Moreover, while RILP-EGFP was associated with CD63-mCherry<sup>+</sup> Flag-TrkA endosomes in distal axons, Rab7 depletion abolished this association, suggesting that recruitment of RILP to TrkA MVBs is Rab7 dependent (Figure S3.3D). Strikingly, in neurons expressing an shRNA against RILP, retrograde TrkA transport was severely compromised (Figure 3.3H, I). Taken together, these findings suggest a Rab7-RILP module associated with TrkA MVBs that mediates long-range retrograde transport of TrkA.

## **Conclusions**

In summary, I demonstrated critical roles of Rab7 in retrograde TrkA transport, signaling and survival in vitro and in vivo. My findings support the following model: newly internalized NGF-TrkA complex is sorted into Rab5<sup>+</sup> early endosomes in distal axons. TrkA early endosomes then mature into TrkA MVBs that are Rab7<sup>+</sup> and CD63<sup>+</sup>. Rab7 recruits RILP to TrkA MVBs, which enables them to undergo long-range dynein-dependent transport along microtubules from distal axons to cell bodies. Therefore, a Rab7-RILP module associated with TrkA endosomes is essential for TrkA MVB to become transport competent and thus transmission of retrograde NGF signals in developing neurons. The requirement of Rab7 in both sympathetic and sensory neurons suggests that this could be a general mechanism of axonal transport.

**Figure 3.1. Rab7 mediates survival and synaptogenesis of sympathetic ganglia *in vivo* and retrograde NGF/TrkA signaling and survival *in vitro*.**

(A)  $Rab7^{+/+}; TH^{2a-CreER}$  and  $Rab7^{ff}; TH^{2a-CreER}$  mice were treated with tamoxifen at E14 to induce Cre expression. SCGs were harvested at P0 or P7 and levels of Rab7 protein were assessed by immunoblot.

(B) Sympathetic neurons grown in mass culture were infected with a virus expressing either a control shRNA or an shRNA against Rab7. Cells were harvested 72 hr post-infection and levels of Rab7 were assessed by immunoblot. Rab7 shRNA expression led to ~80% decrease in Rab7 protein levels.

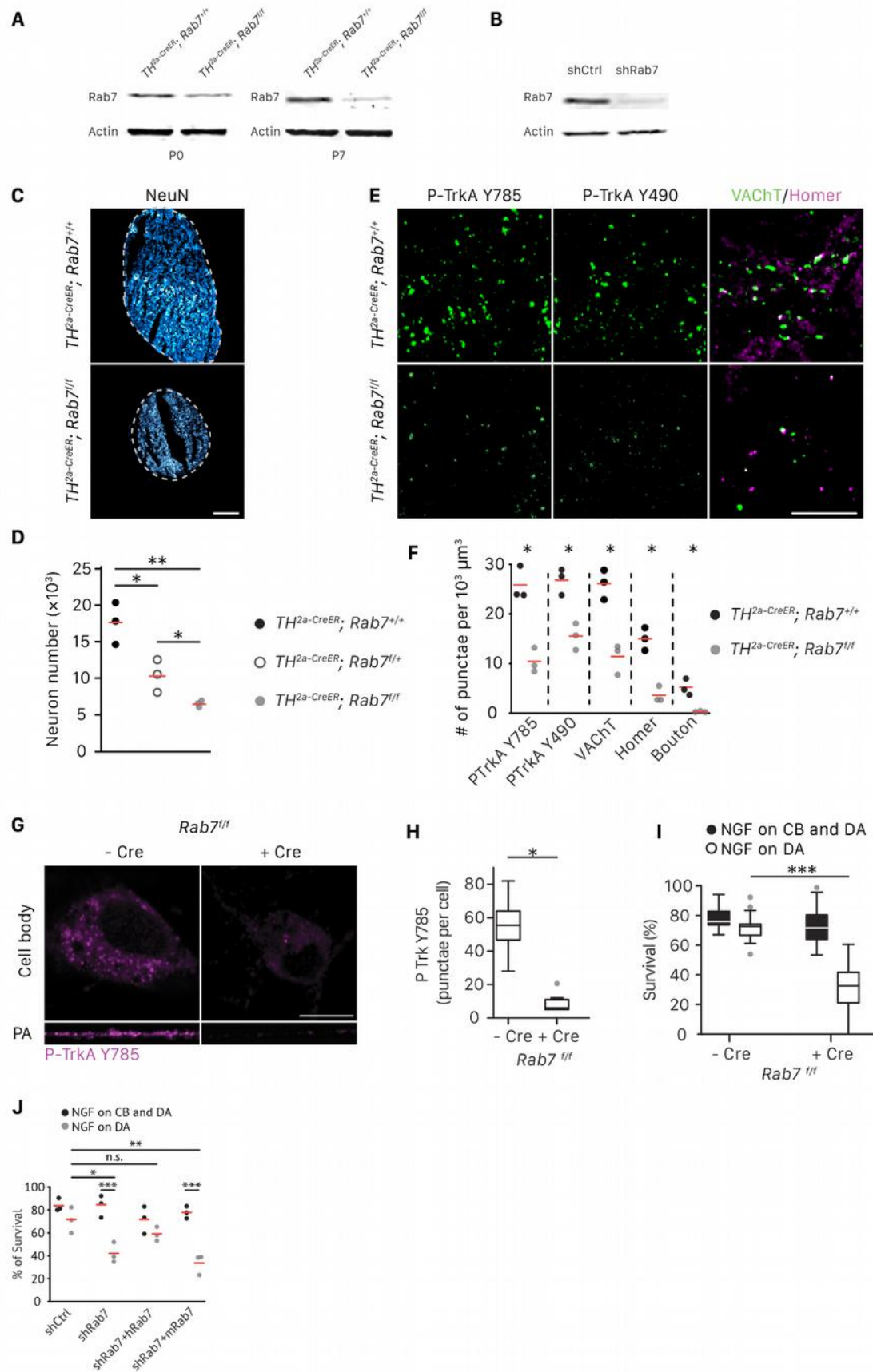
(C, D) Neuronal cell counts of SCGs from  $Rab7^{+/+}; TH^{2a-CreER}$ ,  $Rab7^{ff}; TH^{2a-CreER}$  and  $Rab7^{ff}; TH^{2a-CreER}$  mice at P7. Tamoxifen was administered at E14 (0.5 mg) to induce Cre expression (n = 3). Scale bar: 100  $\mu$ m.

(E, F)  $Rab7^{ff}; TH^{2a-CreER}$  and  $Rab7^{ff}; TH^{2a-CreER}$  mice were treated with 1 mg tamoxifen at P7 and SCGs were harvested at P14. TrkA signaling was assessed by P-Trk Y490 and Y785 staining. Synaptic organization was assessed by VACHT (green) and Homer (magenta) staining, and VACHT/Homer colocalization (n = 3).

(G-I) Sympathetic neurons harvested from P0  $Rab7^{ff}$  pups were grown in microfluidic chambers and infected with a lentivirus expressing the Cre recombinase. Cells were incubated with NGF in the distal axon compartment and anti-NGF and the caspase inhibitor, BAF, in the cell body compartment for 48 hr, and TrkA signaling was assessed in axons and cell soma was assessed by P-Trk Y785 immunostaining (G, H). Alternatively, retrograde NGF-dependent neuronal survival was assessed (I) (n = 3).

(J) Sympathetic neurons grown in compartmentalized chambers were infected with lentivirus expressing: a control shRNA, an shRNA against Rab7, Rab7 shRNA and human Rab7, or Rab7 shRNA and mouse Rab7. Cells were then grown either in the presence of NGF only in the distal axon compartment, or with NGF in both the distal axon and the cell body compartments. Cell survival was assessed 36-48 hr later (n = 3).

Data are presented in dot plots (D, F, J) or box plots (H, I). \*  $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  by one-way ANOVA followed by Tukey's *post-hoc* correction.

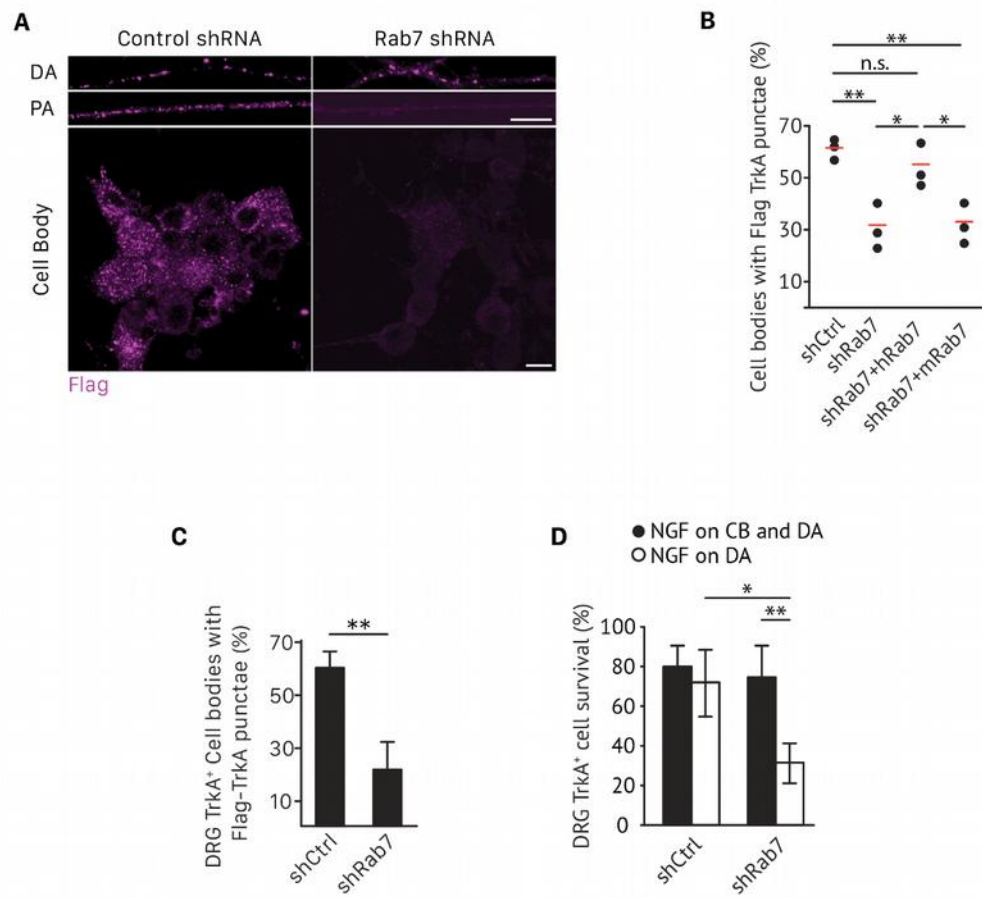


**Figure 3.2. Rab7 is required for retrograde TrkA transport in sympathetic and sensory neurons.**

(**A, B**) The Flag-TrkA transport assay was performed in sympathetic neurons infected with lentivirus expressing either a control shRNA or an shRNA against Rab7. The accumulation of Flag-TrkA punctae in cell bodies, which represent retrogradely transported TrkA, was assessed ( $n = 3$ ). Scale bar: 10  $\mu\text{m}$ .

(**C, D**) DRG primary sensory neurons grown in compartmentalized cultures were infected with lentivirus expressing either a control shRNA or an shRNA against Rab7. 72 hr later, retrograde TrkA transport (**C**) or survival (**D**) was assessed in TrkA<sup>+</sup> neurons ( $n = 3$ ).

Data are presented in dot plot (**B**) or represented as mean  $\pm$  SEM (**C, D**). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  by one-way ANOVA followed by Tukey's *post-hoc* correction.



**Figure 3.3. Recruitment of RILP to TrkA MVBs mediated by Rab7 is required for retrograde TrkA transport.**

(A, B) Colocalization of Flag-TrkA (green) and CD63-mCherry (magenta) in distal axons 60 min post NGF stimulation is comparable between neurons expressing control shRNA or Rab7 shRNA. Quantification is shown in (B).

(C) Colocalization of Flag-TrkA and Rab5 in distal axons 60 min post NGF stimulation is comparable between neurons expressing control shRNA or Rab7 shRNA.

(D) Colocalization between RILP-EGFP and CD63-mCherry<sup>+</sup> Flag-TrkA MVBs in distal axons (60 min post NGF application) in compartmentalized neurons expressing a control shRNA or an shRNA against Rab7. White arrowheads denote RILP/CD63/Flag triple positive endosomes in control axons (left) or CD63/Flag double positive endosomes that are RILP negative (right). Yellow arrowheads show one RILP/CD63/Flag triple positive endosome in Rab7 knockdown axons.

(E) RILP-EGFP (green) co-transport with retrograde Flag-TrkA (magenta) endosomes. Arrowheads denote individual double-labeled endosomes.

(F) Rate of movement of RILP-EGFP<sup>+</sup> TrkA or EGFP-Rab5<sup>+</sup> TrkA endosomes.

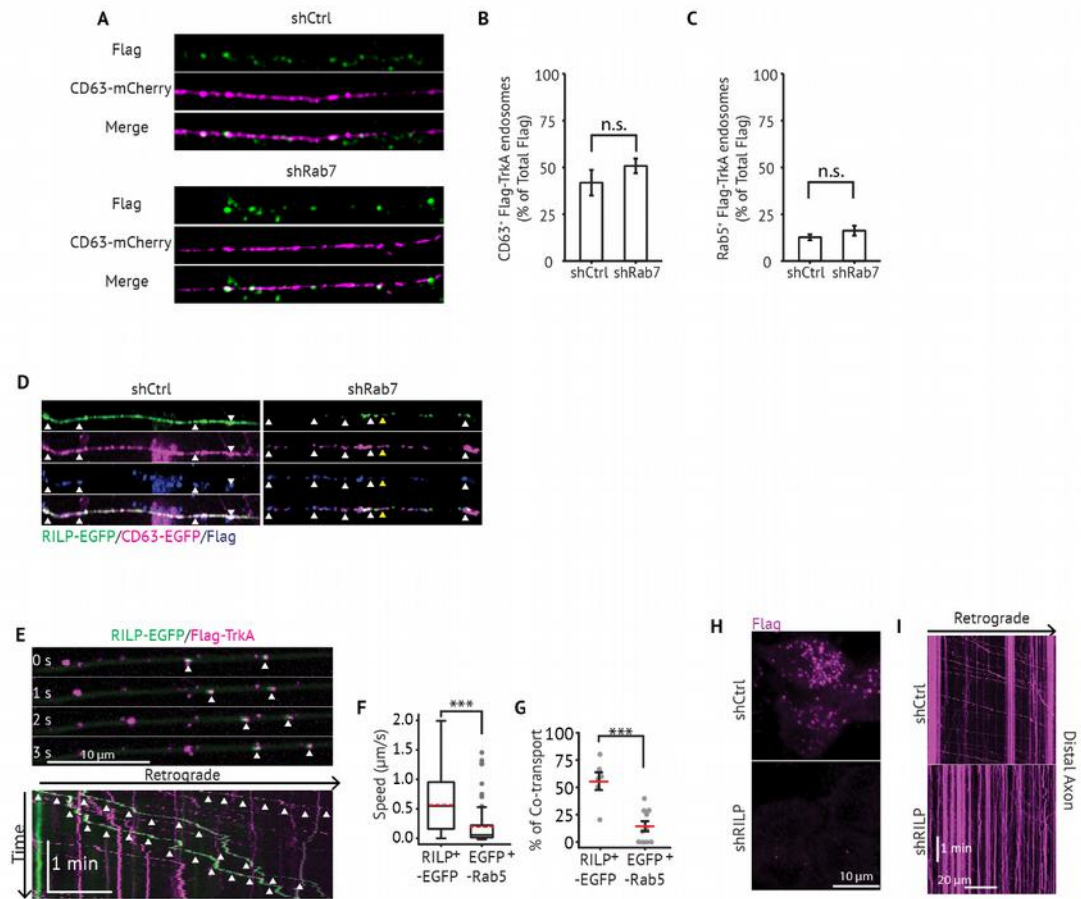
(G) Percentage of co-transport of RILP-EGFP and TrkA or EGFP-Rab5 and TrkA.

(H) Accumulation of retrograde Flag-TrkA endosomes in cell soma in compartmentalized neurons expressing a control shRNA or shRNA against RILP.

(I) Kymograph of Flag-TrkA movement in distal axons in compartmentalized neurons expressing a control shRNA or shRNA against RILP.

Data are represented as mean  $\pm$  SEM (**B, C, G**) or presented in box plot (**F**). or \* $p < 0.05$ ,  
\*\* $p < 0.01$  and \*\*\* $p < 0.001$  by one-way ANOVA followed by Tukey's *post-hoc* correction.





**Chapter 4: Retrogradely transported TrkA<sup>+</sup> endosomes in cell  
bodies evolve from MVBs into simple, single-membrane vesicle  
structures**

## **Introduction**

In findings described in previous chapters, I have established that Rab7<sup>+</sup> MVBs, not Rab5<sup>+</sup> early endosomes, are major carriers of retrograde NGF signals in developing sympathetic neurons. Based on principles of topological organization of endosomes and my immunogold labeling results, it is clear that the C-terminal domain of the TrkA receptor in early endosomes and on the MVB limiting membrane is facing the cytoplasm. On the other hand, TrkA receptors in MVB intraluminal vesicles, which accounts for the majority of the MVB-associated TrkA population, have their C-terminal domain facing the ILV lumen (Hanson and Cashikar, 2012; Katzmann et al., 2002). A key conceptual challenge to the idea that MVBs are TrkA signal carriers, however, is the lack of a plausible mechanism to explain how signals emanating from NGF/TrkA complexes encapsulated within intraluminal vesicles of MVBs are transmitted to the cytoplasm. Here, I investigated this issue by assessing maturation and fate of retrogradely transported TrkA MVBs in cell bodies over time.

## **Materials and Methods**

### **Molecular cloning**

cDNAs of mouse Rab5 and Vps35 were purchased from GE Healthcare. A plasmid containing the APEX2 cDNA was a gift from Alice Ting (Addgene plasmid # 49385). Individual cDNAs or fusion transgenes were cloned into the lentiviral vector FUW by In-Fusion cloning kit (Takara).

### **The pulse-block assay**

The Flag-TrkA assay was performed as above. Nocodazole (10  $\mu$ M) was added to distal axons at indicated time points post NGF application. Cells were fixed at indicated time points and processed for ICC, EM or immunogold labeling as described in previous sections.

## Electron microscopy and APEX2 detection

To detect protein localization by APEX2 reaction, diaminobenzidine (DAB) staining was performed as described previously (Lam et al., 2015): after fixation, cells were incubated in a Tris buffer containing DAB (0.7 mg/ml) and  $\text{H}_2\text{O}_2$  (0.7 mg/ml) for 15-40 minutes until the development of brown reaction product. Cells were then washed extensively to prevent further reaction. Samples were subjected to EM procedures as above. Staining with uranyl acetate, tannic acid and lead citrate was omitted to maximize EM contrast generated by DAB staining.

## Results

From the ultrastructural analysis of retrogradely transported Flag-TrkA<sup>+</sup> endosomes in cell bodies, we observed that the majority of TrkA receptors are localized to the membrane of intraluminal vesicles (ILVs) of TrkA<sup>+</sup> MVBs while a smaller subset is present on the MVB limiting membrane (Figure 3). Based on principles of MVB formation (Hanson and Cashikar, 2012; Katzmann et al., 2002), previous ultrastructural studies of other RTKs (Futter et al., 1996), and our immuno-EM analysis, the topology of TrkA receptors associated with ILVs indicate that its catalytic domain and phosphotyrosine residues are located within the ILV lumen (Figure 2.5B). TrkA receptors in such a configuration are presumably unable to transmit NGF pro-survival signaling cascades to the cytoplasm and nucleus due to the restricted localization of their effector domains.

Therefore, we asked whether the membrane localization of TrkA receptors changes to a position favorable for cytoplasmic signaling following MVB entry into cell bodies. Interestingly, we observed a time-dependent increase in TrkA<sup>+</sup> single-membrane vesicles (SVs) in cell bodies (Figure 4.1A). There are at least two possible explanations for this observation. One possibility is that these TrkA<sup>+</sup> MVBs and SVs are formed in distal axons, and that the TrkA<sup>+</sup> SVs have a

slower transport rate thus explaining their markedly delayed appearance compared to TrkA<sup>+</sup> MVBs. A second possibility is that TrkA<sup>+</sup> SVs are formed *de novo*, within the soma, either directly or indirectly from retrogradely transported TrkA<sup>+</sup> MVBs. To distinguish between these possibilities, we developed a pulse-block TrkA labeling strategy based on the Flag-TrkA EM assay to track the fate of retrogradely transported TrkA<sup>+</sup> MVBs (Figure 4.1B). For this assay, retrograde TrkA transport was allowed to proceed for 25 minutes. Then, all subsequent TrkA axonal transport from distal axons into cell bodies was blocked by applying nocodazole to the distal axon compartment to disrupt microtubules, and thus microtubule-dependent axonal transport, in that compartment. The distribution of Flag-TrkA receptors in different membrane compartments within cell bodies was then assessed by EM at different time points following nocodazole application. The blockade of transport in distal axons by nocodazole was highly effective since it completely blocked accumulation of newly formed Flag-TrkA endosomes within the cell body compartment (Figure 4.1C). In addition, live cell imaging experiments revealed that retrograde axonal movement of Flag-TrkA<sup>+</sup> endosomes in distal axons ceased within 15 to 20 minutes of nocodazole application (Figure 4.1D). Moreover, nocodazole treatment at 25 min post-NGF application to distal axons resulted in sparse accumulation of retrograde TrkA endosomes in cell bodies at the 1hr time point post-NGF treatment and a relatively low, but stable number of TrkA receptors were observed over the subsequent several hours (Figure 4.1E). In contrast, neurons without axonal transport blockade exhibited a dramatic increase in the number of retrograde Flag-TrkA<sup>+</sup> vesicles appearing in cell bodies during the 1 hr time period (Figure 4.1E). Furthermore, nocodazole application to distal axons did not affect microtubule-dependent endosome maturation and trafficking within the cell body compartment (Figure 4.1F, G), indicating effective compartmentalization of transport blockade. These findings

indicate that any change in TrkA<sup>+</sup> endosome ultrastructure in cell bodies at times following addition of nocodazole to distal axons is the result of maturation or evolution of existing endosomes that had arrived prior to nocodazole application, and not newly trafficked endosomes. Thus, this pulse-block Flag-TrkA EM paradigm allowed us to define the dynamics of membrane localization of retrogradely transported TrkA receptors following their arrival to the cell body.

Using this pulse-block TrkA labeling assay, the majority of TrkA receptors observed in cell bodies by EM were found to be associated with one of three types of membrane structures: MVBs, single-membrane vesicles (SVs), and lysosomes (Figure 4.2A). Moreover, the membrane localization of the Flag epitope was in agreement with the principle of endocytosis: The Flag epitope was found on the outer leaflet of ILV membranes and the inner leaflet of limiting membranes and SVs (Figure 4.2B). Consistent with previous experiments, one hour after NGF application to distal axons, and following blockade of newly arriving endosomes, the majority ( $92.8 \pm 3.6\%$ ) of retrograde TrkA<sup>+</sup> endosomes were MVBs (Figure 4.2C, left panel). Among the MVB-resident TrkA receptors,  $68.3 \pm 2.6\%$  were localized to ILVs and  $24.5 \pm 4.7\%$  to the limiting membrane (Figure 4.2C, right panel). Strikingly, the fraction of total cell body Flag punctae found associated with MVBs decreased during the next several hours, and this decrease was coincident with the emergence of Flag-TrkA<sup>+</sup> SVs, which peaked ( $42.4 \pm 8.2\%$ ) at ~5 hours post NGF treatment (Figure 4.2C). The fraction of Flag-TrkA<sup>+</sup> puncta associated with lysosomes remained low during the first 8 hours of blocking the arrival of new endosomes, and by 24 hours post NGF the vast majority of Flag-TrkA receptors were found associated with lysosomes (Figure 4.2C).

To ask whether the MVB-to-SV transition occurs for MVBs carrying other types of cargoes, we assessed maturation of retrogradely transported transferrin endosomes. Transferrin

was internalized in distal axons in a dynamin-dependent manner and colocalized with transferrin receptor, similar to that of NGF/TrkA (Figure 4.3A, B). The majority of newly internalized transferrin and Flag-TrkA are co-localized, suggesting that they can be sorted to the same early endosomes (Figure 4.3C). Interestingly, the level of co-localization decreased over time, indicating that they were later segregated into different vesicular compartments (Figure 4.3C). Moreover, retrogradely transported Flag-TrkA and transferrin remain segregated in cell bodies (Figure 4.3D). Consistent with this, by labeling transferrin and Flag-TrkA with different size gold particles, we found that retrogradely transported transferrin and TrkA were localized to distinct MVBs by EM analysis (Figure 4.3E). Remarkably, in stark contrast to emergence of TrkA<sup>+</sup> SVs in cell bodies, we did not observe formation of SVs for retrograde MVBs carrying transferrin, a ‘non-signaling’ cargo (Figure 4.3F). Instead, the majority of retrogradely transported transferrin proteins were observed in lysosomes as early as the 2hr time point, when TrkA was mostly associated with MVBs and SVs. Therefore, we identified two distinct populations of retrogradely transported MVBs carrying either transferrin or TrkA, and more importantly, only TrkA MVBs gave rise to SVs.

We next asked whether TrkA<sup>+</sup> SVs that emerge in cell bodies are poised for signaling and associated with signaling effectors. SVs were visualized using immuno-gold labeling of P-TrkA and P-PLC $\gamma$  in the pulse-block Flag-TrkA EM paradigm. Indeed, 5hr following NGF application to distal axons, Flag-TrkA receptors localized to the membrane of SVs were observed co-localized with P-TrkA and P-PLC $\gamma$ , with the Flag epitope oriented on the luminal side of the SV membrane and the phospho-tyrosine residues on the cytoplasmic side (Figure 4.4). These observations suggest that TrkA<sup>+</sup> SVs derived from MVBs are signaling competent and capable of transducing TrkA signals within the cell body.

Next we sought to define molecular features of TrkA<sup>+</sup> SVs in cell bodies. To determine whether TrkA<sup>+</sup> SVs in cell bodies of sympathetic neurons are associated with Rab5 following retrograde transport, we determined the extent of co-localization between Flag-TrkA and Rab5 in cell bodies (Figure 4.5A). At the 5hr time point of the pulse-block Flag-TrkA assay, when approximately 40% of retrogradely transported Flag-TrkA<sup>+</sup> endosomes are of a single vesicular nature (Figure 4.1C), fewer than 5% of the Flag punctae in cell bodies were co-localized with Rab5 (Figure 4.5A, B). The extent of co-localization of Flag-TrkA and Rab5 was also assessed by EM (Figure 4.5C-E). To visualize Rab5<sup>+</sup> endosomes by EM, we fused Rab5 with APEX2 (Figure 4.5C), a modified peroxidase that enables visualization of the product of the peroxidase-catalyzed reaction by EM and thus subcellular distribution of APEX2 fusion proteins (Lam et al., 2015). Lentivirus-mediated delivery of APEX2-Rab5 into sympathetic neurons and EM analysis revealed that the majority of newly internalized Flag-TrkA receptors are associated with APEX2-Rab5<sup>+</sup> endosomes ( $67.9 \pm 7.2\%$ ; Figure 4.5D, middle; Figure 4.5E), as predicted. In contrast, and consistent with our immunocytochemical analyses, very few Flag-TrkA punctae were found to be associated with APEX2-Rab5<sup>+</sup> structures at the 5hr time point of the pulse-block Flag-TrkA assay ( $6.4 \pm 2.0\%$ ; Figure 4.5D, right; Figure 4.5E). Taken together, these findings indicate that TrkA<sup>+</sup> SVs that emerge from MVBs in cell bodies are not associated with Rab5, which is a distinguishing feature of early endosomes.

Endocytic pathways downstream of MVBs, other than the well-described degradation pathway, include the recycling pathway and the retromer-dependent transport pathway to the Golgi apparatus (Cullen and Korswagen, 2012; Katzmann et al., 2002). Since TrkA<sup>+</sup> SVs in cell bodies are derived from TrkA<sup>+</sup> MVBs, the TrkA endocytic pathway in cell bodies might engage one or both of these routes. Indeed, we observed some TrkA<sup>+</sup> SVs in close proximity to Golgi, while



others were close to the plasma membrane and exhibited a tubular shape resembling recycling endosomes (Figure 4.5F). To more directly test these possibilities, we assessed the extent of Flag-TrkA co-localization with Rab11 and Vps35, key components of recycling endosomes and the retromer complex, respectively (Figure 4.5G). At the 5hr time point of the pulse-block Flag-TrkA assay, Flag-TrkA<sup>+</sup> endosomes were associated with Vps35 ( $39.8 \pm 12.5\%$ ) and to a lesser extent Rab11 ( $13.0 \pm 6.6\%$ , Figure 4.5B, G). Interestingly, nearly all Rab11<sup>+</sup> Flag-TrkA endosomes were also Vps35<sup>+</sup> ( $10.3 \pm 5.7\%$ , Figure 4.5B, G). We confirmed the Vps35 identity of TrkA<sup>+</sup> SVs by APEX2-Vps35/Flag EM double labeling ( $58.9 \pm 8.3\%$  of total Flag-TrkA SVs, Figure 4.5H-J). Therefore, a significant portion of TrkA<sup>+</sup> SVs exhibit a molecular feature of retromer complexes, and a subset may include Rab11<sup>+</sup> recycling endosomes.

## Conclusions

Because the transport blockade prevented TrkA<sup>+</sup> endosomes from entering cell bodies in this pulse-block paradigm, the most parsimonious explanation of the source of the TrkA<sup>+</sup> SV population during the course of these experiments is that they emerge *de novo* within cell bodies. Since the vast majority of newly arrived Flag-TrkA<sup>+</sup> endosomes at the start of the transport blockade are MVBs, the Flag-TrkA<sup>+</sup> SVs are therefore derived, either directly or indirectly, from TrkA<sup>+</sup> MVBs. Remarkably, in stark contrast to emergence of TrkA<sup>+</sup> SVs in cell bodies, we did not observe formation of SVs for retrograde MVBs carrying transferrin, a ‘non-signaling’ cargo (Figure 5F). These observations indicate that the membrane localization of retrogradely transported TrkA, and indeed the ultrastructural properties of TrkA<sup>+</sup> endosomes, changes following TrkA<sup>+</sup> endosome entry into the cell body. Moreover, retrograde TrkA receptors localized to the membrane of SVs are topologically organized in a manner that, in principle, enables downstream effector association and signaling within the cytoplasm. Thus, Flag-TrkA<sup>+</sup>

SVs emerge from TrkA<sup>+</sup> MVBs in cell bodies, and this transformation reveals a plausible mechanism of retrograde NGF signal transduction within the cytoplasm of cell bodies.

**Figure 4.1. Nocodazole treatment effectively blocks microtubule-dependent axonal trafficking and retrograde Flag-TrkA transport.**

(A) Quantification of retrograde Flag-TrkA localization in MVB, SV and lysosome in cell bodies over time ( $n = 4$ , > 200 gold particles scored for each time point).

(B) Schematic of the pulse-block assay. The Flag-TrkA assay is performed in *TrkA<sup>Flag</sup>* sympathetic neurons cultured in compartmentalized microfluidic chambers using pre-conjugated anti-Flag antibody and Protein A-5 nm gold. Nocodazole is applied to distal axons 25 minutes post NGF application to block retrograde transport. Neurons are fixed at indicated time points and processed for EM.

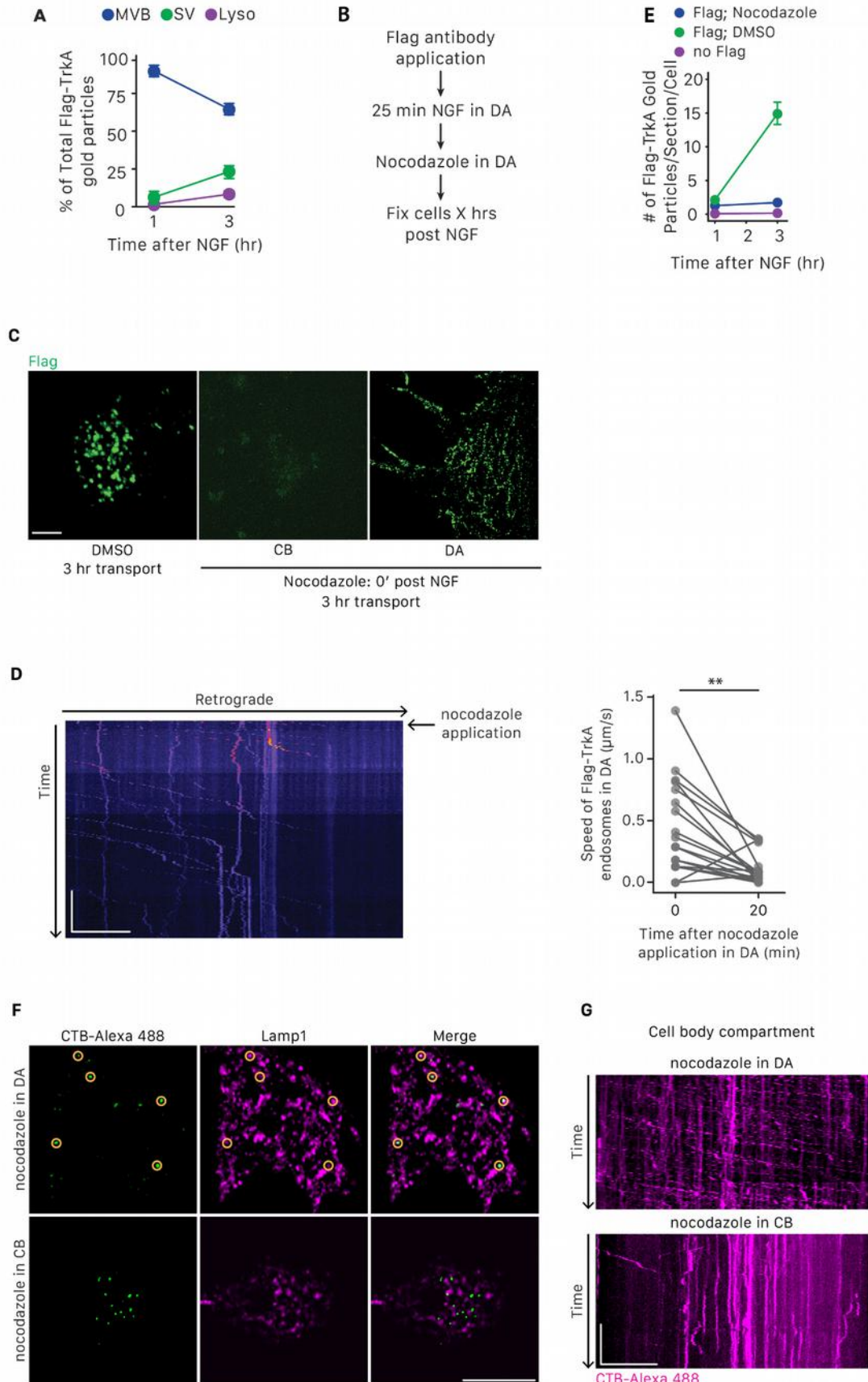
(C) The Flag-TrkA assay was performed in compartmentalized sympathetic neurons with DMSO or nocodazole (10  $\mu$ M) applied at the time of NGF application in DA. Accumulation of Flag-TrkA punctae in cell bodies and distal axons was assessed 3 hr post NGF application ( $n = 3$ ). Compared to the vehicle control, very few, if any, Flag punctae were observed in nocodazole treated cells. Scale bar: 20  $\mu$ m.

(D) The Flag-TrkA assay was performed in compartmentalized sympathetic neurons with nocodazole (10  $\mu$ M) applied 25 minutes post NGF application in DA. Movement of retrograde Flag-TrkA endosomes was monitored by live imaging in the middle grooves of microfluidic chambers ( $n = 3$ ). A representative kymograph is shown (left panel). The rate of movement of individual Flag-TrkA endosomes in distal axons at the time of nocodazole application and 20 minutes afterwards was measured (Right panel). Retrograde movement halted ~15 minutes post nocodazole application. Scale bar: 20  $\mu$ m; 5 minutes.

(E) The Flag-TrkA assay was performed in WT neurons, *TrkA<sup>Flag</sup>* neurons treated with either DMSO or nocodazole in distal axons (25 min post NGF). Cells were fixed at indicated time points and processed for EM. The number of gold particles per section per cell was quantified.

(F, G) Alexa 488 labeled CTB was applied to the cell body compartment of compartmentalized neuronal culture in the presence of nocodazole either in the cell body or distal axon compartment. The endocytic trafficking of CTB-Alexa488 to lysosomes (F) and axonal movement of CTB (G) were assessed. Scale bar: 10  $\mu$ m (F). 10  $\mu$ m; 1 min (G).

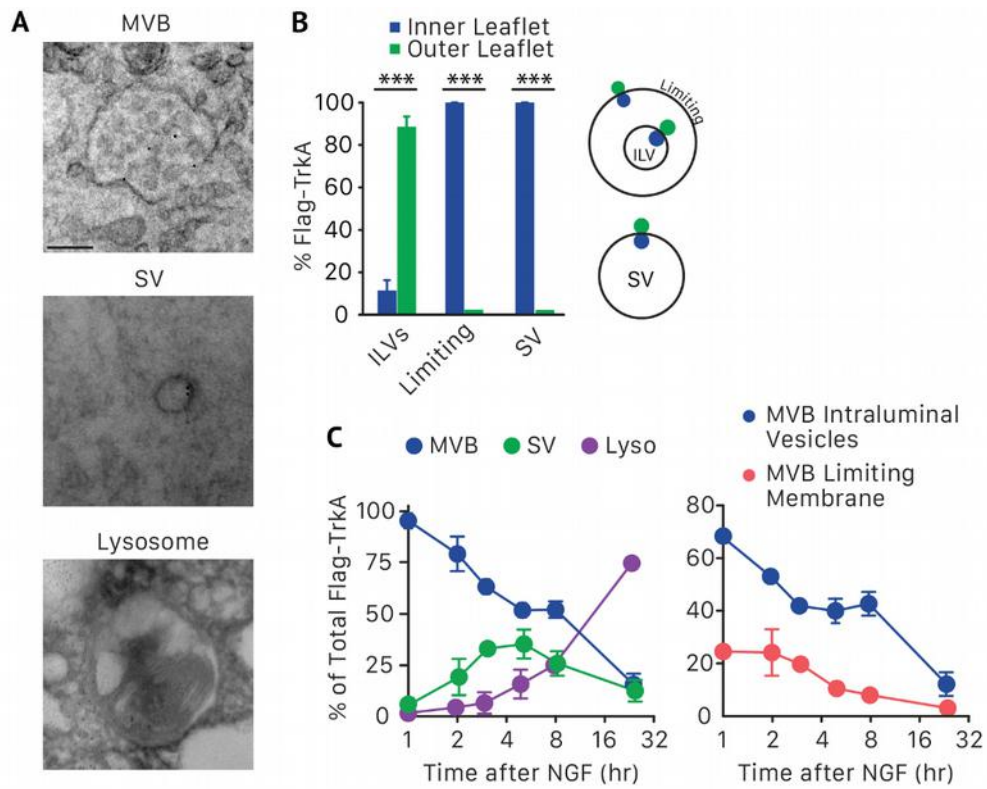
Data are represented as mean  $\pm$  SEM. \*\*  $P < 0.01$  using a two-tailed paired Student's  $t$  test.



**Figure 4.2. Retrogradely transported TrkA<sup>+</sup> endosomes within cell bodies evolve from MVBs into simple, single-membrane vesicle structures.**

(A, C) The pulse-block assay was performed and the distribution of retrogradely transported Flag-TrkA gold particles in MVBs, single-membrane vesicle structures (SVs) and lysosomes within the cell body over time was assessed (C). Representative images of retrograde Flag-TrkA in each of the three membrane compartments are shown in (A).

(B) The pulse-block assay was performed and membrane topology of the Flag epitope was assessed. A schematic of the inner- or outer-leaflet position for membrane of ILVs, the limiting membrane and SVs is shown on the right.



**Figure 4.3. Retrogradely transported transferrin and Flag-TrkA are sorted in distinct MVBs.**

(A) Internalized transferrin-Alexa 546 was co-localized with transferrin receptor in distal axons of compartmentalized sympathetic neurons. Scale: 10  $\mu$ m.

(B) Internalization of transferrin-Alexa 546, but not BSA, in distal axons of compartmentalized sympathetic neurons was abolished with treatment of Dyngo, a dynamin inhibitor, exclusively to distal axons. Scale: 10  $\mu$ m.

(C) Internalized transferrin-Alexa 546 and Flag-TrkA were co-localized in distal axons of compartmentalized sympathetic neurons after 5 min, but not 30 min, NGF stimulation. Scale: 10  $\mu$ m.

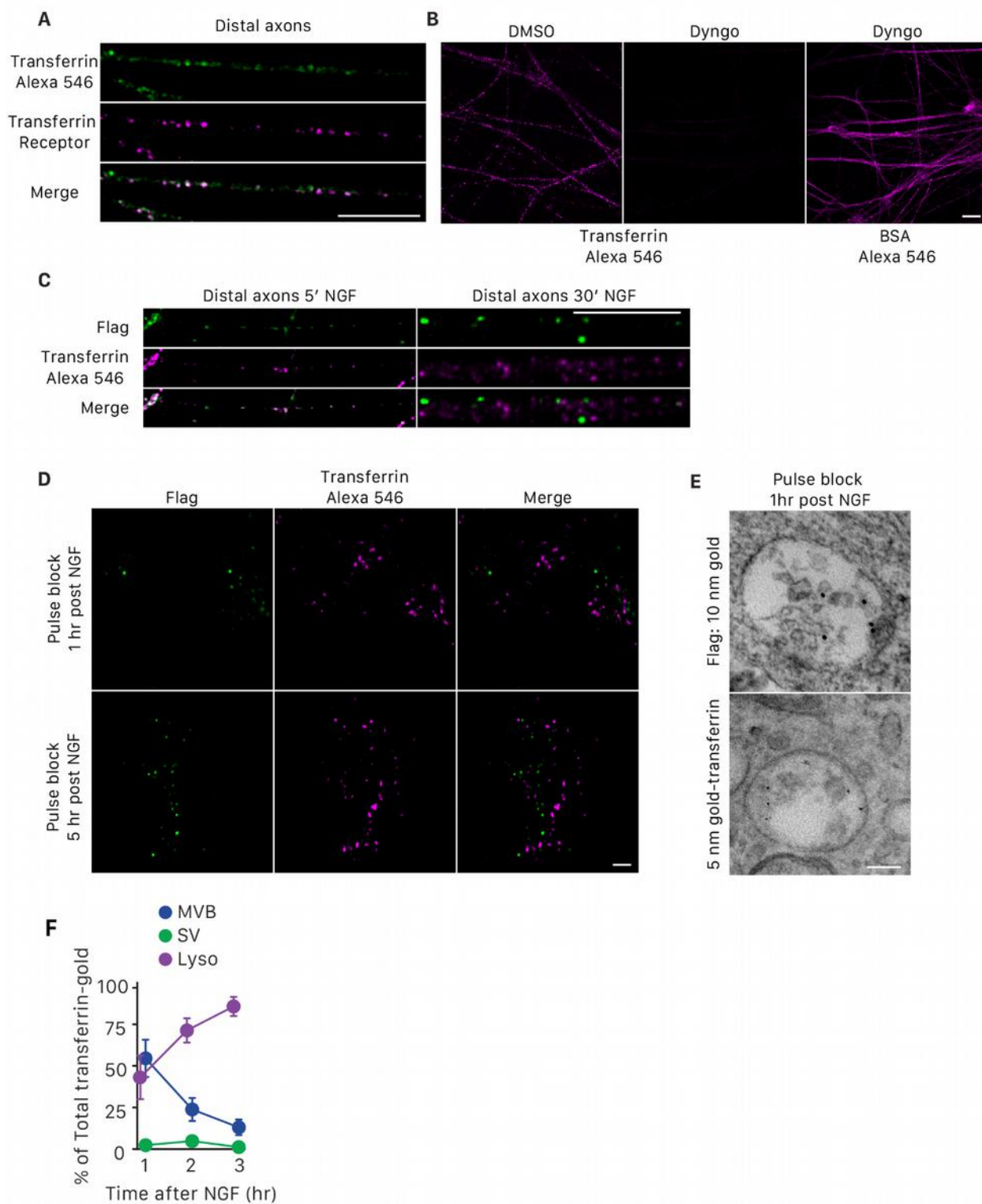
(D) Retrogradely transported Flag-TrkA and transferrin-Alexa 546 were not co-localized in cell bodies. Distal axons of compartmentalized sympathetic neurons were incubated with Flag antibody and transferrin-Alexa 546. The pulse-block assay was performed. Cells were fixed 1hr or 5hr post NGF application and assessed by immunostaining.

(E) Distal axons of compartmentalized sympathetic neurons were incubated with Flag antibody conjugated to Protein A-10nm gold and transferrin-5nm gold. The pulse-block assay was performed. Cells were fixed 1hr post NGF application assessed by EM.

(F) Sympathetic neurons in compartmentalized cultures were incubated with transferrin-gold (6 nm) in distal axons and the pulse-block assay was performed. The distribution of retrograde transferrin-gold in MVBs, SVs and lysosomes was assessed by EM. Over 150 endosomes were counted for each condition at each time point in 4 independent experiments. Scale bar: 100 nm.

Data are represented as mean  $\pm$  SEM.





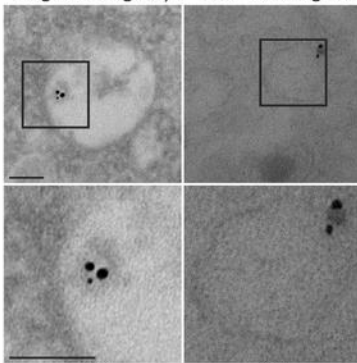
**Figure 4.4. TrkA<sup>+</sup> single vesicles formed *de novo* in cell bodies after retrograde transport are signaling competent.**

(A) The pulse-block assay was performed in compartmentalized sympathetic neurons using pre-conjugated anti-Flag antibody with 5 nm gold secondary antibody. Cells were fixed 5 hr post NGF application and immunogold-labeled for P-Trk or P-Plcγ (10 nm). Shown are representative images of an MVB and SV containing retrograde Flag-TrkA gold particles that are juxtaposed to P-Trk or P-PLCγ. High magnification images of the boxed areas are shown in the bottom panel. The percentage of these signaling competent MVBs and SVs in cell bodies was quantified (n = 3). Scale bar: 100 nm.

Data are presented in dot plot.

**A**

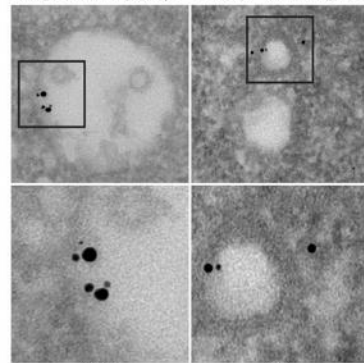
Flag: 5 nm gold; P-Trk: 10 nm gold



MVB

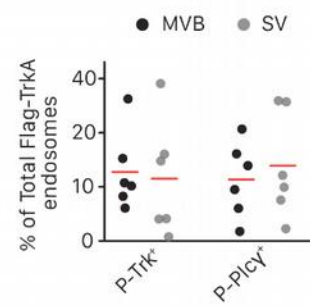
SV

Flag: 5 nm gold; P-PLCγ: 10 nm gold



MVB

SV



**Figure 4.5. TrkA<sup>+</sup> single vesicles formed *de novo* in cell bodies after retrograde transport are not Rab5<sup>+</sup> early endosomes.**

(A, B) The pulse-block assay was performed in compartmentalized sympathetic neurons and colocalization between Flag-TrkA (green) and Rab5 (magenta) in cell bodies was assessed 5 hr post NGF application. Insets show magnification of the boxed areas. Quantification is shown in (B) (n = 4). Scale bar: 5  $\mu$ m and 1  $\mu$ m (inset).

(C) Characterization of APEX2-Rab5. Sympathetic neurons in mass culture were infected with a virus expressing APEX2-Rab5. 48 hr post-infection, cells were fixed, subjected to DAB staining and processed for EM. For each construct, a representative EM micrograph is shown with an infected cell and an adjacent non-infected cell. Insets show higher magnification of the boxed areas. No overt abnormality with respect to cellular organization and vesicle morphology and localization was observed. Notice the overall higher contrast exhibited in cells expressing APEX2. APEX2 signals were enriched around endosomal membranes as seen from the images with higher magnification. Scale bar: 5  $\mu$ m; 100 nm (inset).

(D, E) Sympathetic neurons grown in mass culture were infected with a virus expressing APEX2-Rab5. The Flag-TrkA assay was performed using pre-conjugated anti-Flag antibody with Protein A-5 nm gold secondary antibody and cells were fixed 5 minutes post NGF stimulation. DAB staining was performed and cells were processed for EM. The extent of gold particles associated with APEX2<sup>+</sup> endosomes in cell bodies was assessed. APEX2<sup>+</sup> endosomes were identified based on the dark staining associated with endosomal membranes (arrowheads), compared to the lack of contrast in non-infected cells (left panel). In the right panel, the pulse-block assay was performed in compartmentalized sympathetic neurons expressing APEX2-Rab5. Cells were fixed 5 hr post NGF stimulation and the extent of gold particles resided in APEX2<sup>+</sup>

endosomes was assessed. Arrows denote endosomes containing Flag-TrkA and arrowheads denote APEX2<sup>+</sup> endosomes. Quantification is shown in **(E)** (n = 3). Scale bar: 100 nm.

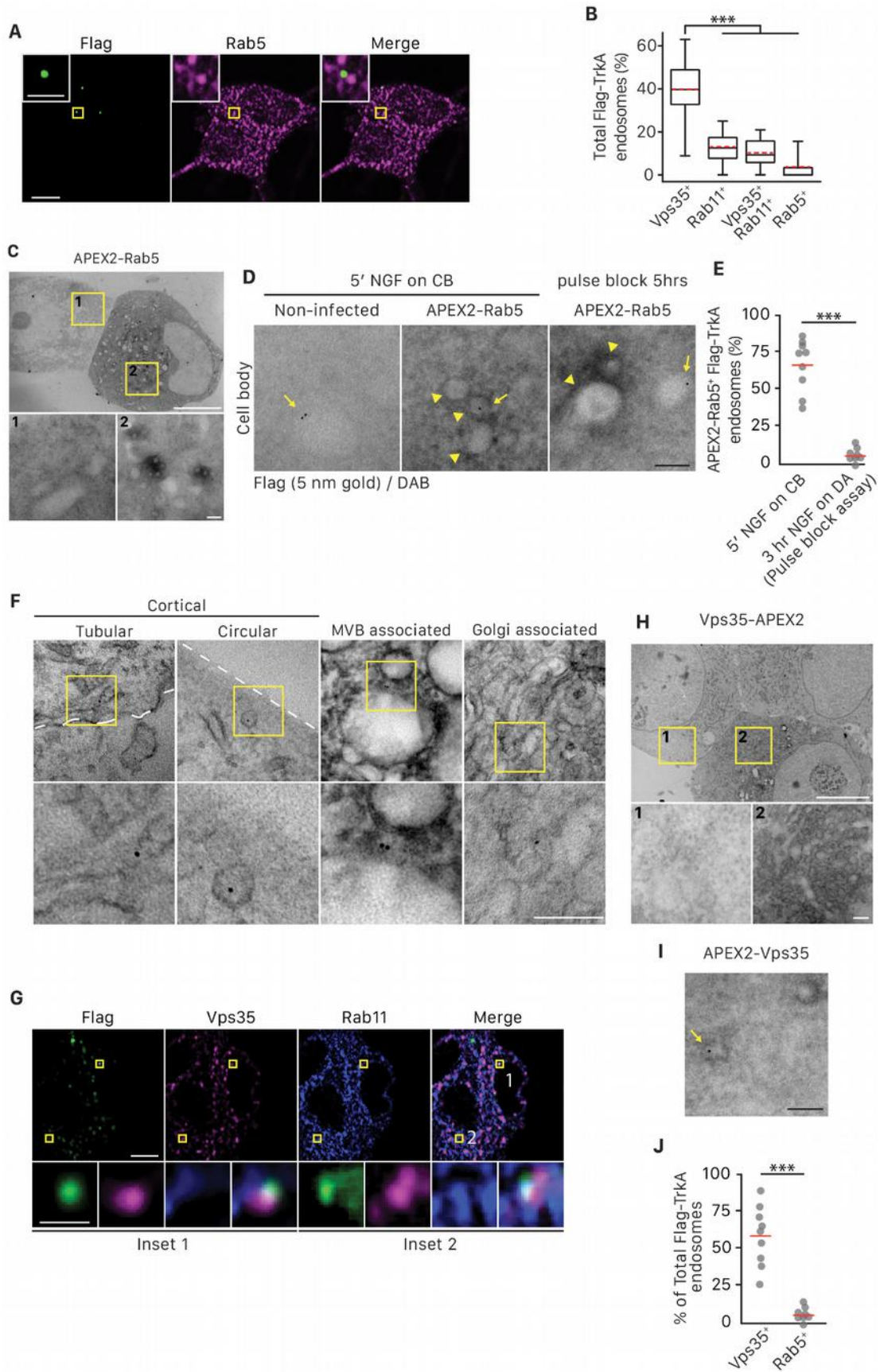
**(F)** The pulse-block assay was performed in compartmentalized sympathetic neurons. Cells were fixed 5 hr post NGF application and processed for EM. Shown are examples of Flag-TrkA<sup>+</sup> single vesicles that have distinct morphology, subcellular localization and organelle association. Left to right: a tubular shaped TrkA SV that is close to the plasma membrane (white dashed contour); a circular shaped TrkA SV that is close to the plasma membrane (white dashed contour); a circular shaped TrkA SV that is associated with an MVB; and a tubular shaped TrkA SV that is associated with a Golgi apparatus. Magnified images of boxed areas are shown in the lower panel. Scale bar: 100 nm.

**(G)** The pulse-block assay was performed in compartmentalized sympathetic neurons and colocalization between Flag-TrkA (green), Vps35 (magenta) and Rab11 (blue) in cell bodies was assessed at 5 hours post NGF application. Insets show magnification of the boxed areas. Quantification is shown in **(B)** (n = 4). Scale bar: 5  $\mu$ m and 1  $\mu$ m (inset).

**(H)** Characterization of APEX2-Vps35.

**(I, J)** The pulse-block assay was performed in compartmentalized sympathetic neurons expressing APEX2-Vps35. Cells were fixed 5 hr post NGF stimulation and the extent of gold particles resided in APEX2<sup>+</sup> endosomes was assessed (n = 3). A representative EM image is shown in **(I)**. Quantification is shown in **(J)**. Scale bar: 100 nm.

Data are presented in box plot **(B)** or dot plots **(E, J)**. \*\*\*p < 0.001 by one-way ANOVA followed by Tukey's *post-hoc* correction **(B)** or two tailed unpaired Student's *t* test **(E, J)**.



**Chapter 5: TrkA kinase activity directs the fate of retrogradely  
transported TrkA<sup>+</sup> endosomes**

## **Introduction**

Retrograde NGF signal transmission requires an active NGF-TrkA signaling pathway. Autophosphorylation of TrkA and active PI3K signaling in both distal axons and cell bodies, but not middle axons, are necessary for retrograde TrkA transport, CREB phosphorylation, retrograde survival and PSD formation. PLC $\gamma$  activity appears to be necessary for TrkA internalization. RAS-MAPK activity in cell bodies, but not distal axons, is also required for retrograde NGF survival. However, less is known about the contribution of each of these signaling modules during formation and transport of TrkA signaling endosomes and their maturation in distal axons and in cell soma. In this chapter, I addressed this question by pharmacologically inhibiting TrkA kinase activity associated with TrkA endosomes and assessing endosome maturation and fate.

## **Materials and Methods**

### **Reagents and Antibodies**

K252a, 1NMPP1 and DMSO were obtained from EMD Millipore. 6 nm gold-conjugated transferrin, CTB or BSA were obtained from Electron Microscopy Sciences. Protein A-5nm/10nm gold antibodies were made by The Harvard Medical School EM Facility and used at a dilution of 1:50.

### **Molecular cloning, transfection and lentiviral infection**

The Flag-TrkB/A-WT and Flag-TrkB/A-F592A constructs were synthesized (Biomatik) and subcloned into the FUW vector. The TrkB/A chimeric receptor comprises the extracellular domain of TrkB (nucleotide 1-1242) and the transmembrane and intracellular domains of TrkA (nucleotide 1156-2400).



DNA plasmids were transfected into neuronal cells on DIV 3 using Lipofectamine 2000 (1 µg DNA: 4 µl Lipofectamine per well).

Lentivirus was generated, harvested and concentrated as previously described (Salmon and Trono, 2007). Neuronal cultures were infected on DIV 3 and experiments were performed 48~72 hr later.

#### The pulse-block kinase assay

Compartmentalized sympathetic neurons were infected with lentivirus expressing either Flag-TrkB/A-F592A or Flag-TrkB/A-WT. The Flag-TrkA assay was performed as described with the following modification: cells were stimulated with BDNF instead of NGF, and either DMSO or the TrkA F592A kinase activity inhibitor 1NMPP1 (500 nM) was applied to the cell body compartment at varying time points as indicated during the course of the experiment. The cells were then subjected to ICC or TEM as above.

## Results

How are MVB, SV and lysosome membrane dynamics regulated following arrival of TrkA<sup>+</sup> MVBs in cell bodies, and to what extent does TrkA signaling control this process? To address this, we used a chemical genetic strategy to manipulate TrkA kinase signaling in a compartmentalized manner. For this purpose, we generated a Flag-TrkB/A-F592A expression construct (Figure 5.1A). This construct encodes a chimeric receptor consisting of the TrkB extracellular domain and the TrkA transmembrane and intracellular domains so that BDNF rather than NGF can activate TrkA intracellular signaling in transduced cells (Figure 5.1A). Moreover, the TrkB/A fusion protein contains the Flag epitope tag for detecting retrogradely transported receptors by both light microscopy and EM, as done using neurons harvested from *TrkA<sup>Flag</sup>* mice.

In addition, the construct contains the F592A point mutation within the TrkA kinase domain ATP binding pocket, which renders TrkA catalytic activity sensitive to inhibition by the small molecule 1NMPP1 (Figure 5.1A) (Chen et al., 2005).

As expected (Chen et al., 2005), expression of Flag-TrkB/A-F592A enabled survival of sympathetic neurons treated with BDNF acting exclusively on distal axons (Figure 5.1C). Furthermore, exposure of Flag-TrkB/A-F592A infected neurons to BDNF in the absence of NGF was sufficient to promote the generation and transport of Flag-TrkB/A-F592A<sup>+</sup> signaling endosomes, as seen by an increase in P-Trk immunoreactivity by both light microscopy and EM (Figure 5.1D and E). Importantly, Flag-TrkB/A-F592A autophosphorylation was eliminated upon exposing neurons to 1NMPP1 (Figure 5.1D and E). The elimination of P-Trk signaling in the context of BDNF stimulation also confirmed the lack of activation of endogenous TrkA receptors, which are insensitive to 1NMPP1. Therefore, the Flag-TrkB/A-F592A construct enables both immunodetection of retrogradely transported Flag-TrkB/A-F592A<sup>+</sup> endosomes and precise manipulation of its associated TrkA kinase activity.

To test whether TrkA kinase activity is required for membrane redistribution of retrogradely transported TrkA receptors following TrkA<sup>+</sup> endosome entry into cell bodies, we used the pulse-block Flag-TrkA assay and sympathetic neurons that express either Flag-TrkB/A-F592A or Flag-TrkB/A-WT (lacking 1NMPP1 sensitivity) and treated with either 1NMPP1 or the vehicle DMSO applied to the cell body compartment (Figure 5.1B). Distal axons of these neurons were exposed to BDNF for 5hr, and the extent of lysosomal localization of retrogradely transported receptors was then assessed by Flag/Lamp1 co-localization (Figure 5.2A). Interestingly, in cells expressing Flag-TrkB/A-F592A and treated with 1NMPP1 applied to their cell bodies, we observed a dramatic increase in Lamp1<sup>+</sup> Flag endosomes compared to the three

control conditions (Figure 5.2A and B). These findings indicate that TrkA kinase signaling in cell bodies prevents precocious sorting of retrograde TrkA<sup>+</sup> endosomes to lysosomes.

To further explore the role of TrkA kinase activity in endosome sorting within cell bodies, we analyzed the relative amounts of retrogradely transported Flag-TrkB/A-F592A in association with MVBs, SVs and lysosomes by EM (Figure 5.2B and C). Neurons expressing Flag-TrkB/A-F592A and treated with either DMSO or 1NMPP1 in the cell body compartment exhibited a similar pattern of TrkA membrane localization at the 1hr time point, with the majority of Flag-TrkB/A-F592A associated with MVBs (Figure 5.2C). However, at later time points, neurons exposed to 1NMPP1 on cell bodies exhibited a much lower fraction of Flag-TrkB/A-F592A<sup>+</sup> MVBs, virtually no Flag-TrkB/A-F592A<sup>+</sup> SVs, and a much higher percentage of Flag-TrkB/A-F592A<sup>+</sup> lysosomes, compared to DMSO control-treated neurons (Figure 5.2B and C). These observations indicate that TrkA kinase activity is necessary for the proper distribution of retrogradely transported TrkA within MVBs and SVs and the prevention of premature lysosomal sorting following endosome arrival to cell bodies.

Finally, we asked whether the distribution of TrkA in MVBs, SVs and lysosomes is controlled by local TrkA signaling associated with endosomes themselves or whether TrkA signals emanating from other platforms, i.e. *in trans* from other TrkA endosomes or the plasma membrane, can control the fate of retrogradely transported TrkA endosomes. For this, we compared the fate of retrogradely transported endosomes that contain either TrkA or non-TrkA cargoes, including transferrin, bovine serum albumin (BSA), and Cholera toxin subunit B (CTB), by EM analysis (Figure 5.2B-E). As for TrkA<sup>+</sup> endosomes, the majority of retrogradely transported transferrin<sup>+</sup>, BSA<sup>+</sup> or CTB<sup>+</sup> endosomes newly arrived within cell bodies were MVBs (Figure 5.2B-E). In dramatic contrast to TrkA<sup>+</sup> endosomes, however, very few, if any transferrin<sup>+</sup>,

BSA<sup>+</sup> and CTB<sup>+</sup> endosomes redistributed to SVs during the subsequent several hours. Rather, the vast majority of these non-TrkA MVBs were rapidly sorted into lysosomes (Figure 5.2B-E). This rapid sorting of transferrin<sup>+</sup>, BSA<sup>+</sup> and CTB<sup>+</sup> endosomes to lysosomes was independent of 1NMPP1 treatment, and was similar to the rapid sorting of TrkA<sup>+</sup> endosomes to lysosomes observed in neurons expressing Flag-TrkB/A-F592A and receiving 1NMPP1 treatment.

Lastly, we asked whether TrkA signaling acting *in trans* can control the fate of retrogradely transported TrkA<sup>+</sup> MVBs. To address this possibility, we conducted similar Flag-TrkB/A-F592A experiments as those described above, although these were performed in the presence of NGF applied to the cell body compartment to activate endogenous TrkA signaling. Interestingly, NGF application partially rescued premature lysosome sorting of retrograde Flag-TrkB/A-F592A<sup>+</sup> MVBs in neurons treated with 1NMPP1 (Figure 5.2F). However, NGF application directly to cell bodies did not prevent the rapid sorting of retrogradely transported transferrin-containing endosomes to lysosomes (Figure 5.2F). Thus, the fate of retrogradely transported MVBs within cell bodies is dependent on the nature and signaling activity of its cargo. TrkA kinase signaling promotes the emergence of TrkA<sup>+</sup> SVs from TrkA<sup>+</sup> MVBs and protects or delays these endosomes from acquiring a lysosomal fate. Moreover, TrkA receptors can signal *in trans*, albeit it to a lesser extent than endosomal TrkA signals acting *in cis*, to influence the fate of retrograde TrkA<sup>+</sup> signaling endosomes and prevent their lysosomal targeting and degradation.

## Conclusions

In summary, by utilizing a chimeric TrkA receptor that allows for inhibition of TrkA kinase activity in TrkA endosomes, I showed that active TrkA within retrograde TrkA endosomes is required for the formation of TrkA SVs and prevention of lysosomal fusion, both of which

processes augment and sustain NGF signaling transduction. Furthermore, NGF signaling from other compartments of the cell body partially rescued these deficits. On the other hand, retrograde MVBs carrying ‘non-signaling’ cargoes did not give rise to SVs and were fused with lysosomes rapidly and their dynamics were not influenced by cell body-derived NGF signals. Therefore, the nature and signaling state of the cargo critically regulate maturation and fate of retrograde MVBs.

**Figure 5.1. Expression of Flag-TrkB/A-F592A allows specific activation and inhibition of TrkA kinase activity within endosomes.**

(A) Schematic of the Flag-TrkB/A-F592A construct.

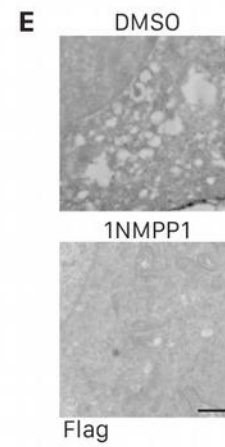
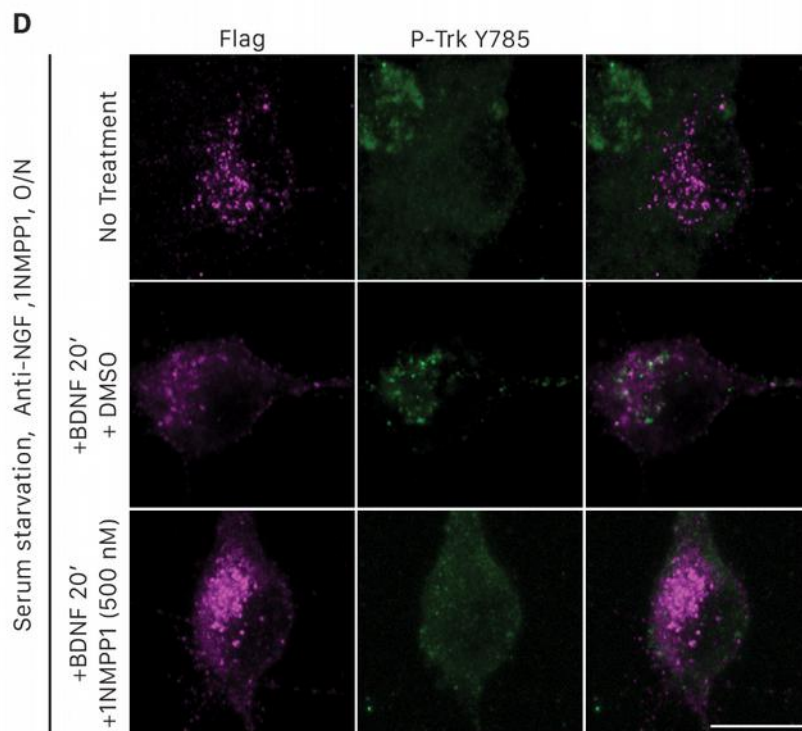
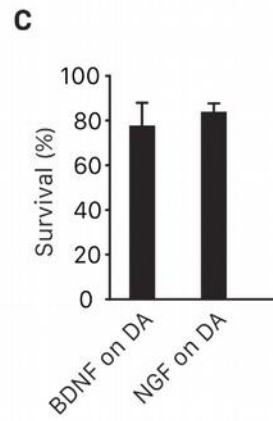
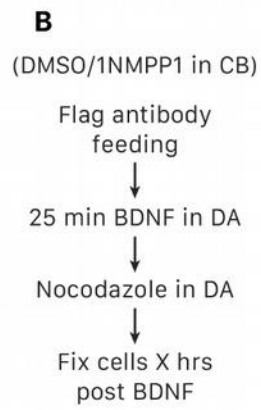
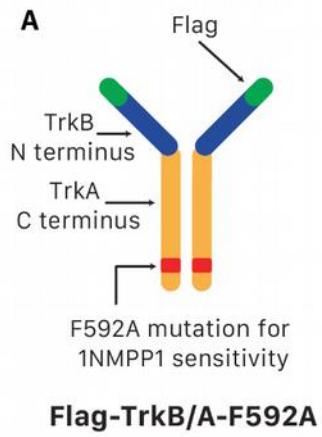
(B) Schematic of the pulse-block kinase assay.

(C) Compartmentalized sympathetic neurons expressing Flag-TrkB/A-F592A were cultured in conditions in which distal axons were exposed to BDNF or NGF while the cell body compartment was neurotrophin-deprived. Neuronal survival was assessed 48 hr later. Neurons expressing Flag-TrkB/A-F592A can rely on BDNF applied to distal axons as the sole source of survival signal, indicating that the modified receptor can be activated by BDNF and the retrograde survival signal can be transmitted to cell bodies (n = 3).

(D) WT neurons in mass culture expressing Flag-TrkB/A-F592A virus were serum- and neurotrophin-starved overnight and then incubated with no neurotrophin, BDNF and DMSO, or BDNF and 500 nM 1NMPP1. 20 minutes later, cells were salt/acid stripped and Flag-TrkB/A-F592A internalization and activation was assessed by Flag (magenta) and P-Trk Y785 (green) immunostaining (n = 3). The lack of P-Trk signals in the BDNF/1NMPP1 treated cells suggest that 1NMPP1 treatment is efficacious in inhibiting TrkA F592A kinase activity and BDNF stimulation does not lead to activation of endogenous TrkA receptors, whose kinase activity is 1NMPP1 insensitive. Scale bar: 10  $\mu$ m.

(E) WT neurons in mass culture expressing Flag-TrkB/A-592A were serum- and neurotrophin-starved overnight and subsequently stimulated with BDNF in the presence of DMSO or 1NMPP1 for 20 minutes. Cells were then fixed and processed for P-Trk Y785 immunogold

labeling. 1NMPP1 treatment eliminated the majority of P-Trk signal as seen in the EM micrograph. Scale bar: 1  $\mu\text{m}$ .





**Figure 5.2. TrkA kinase activity within endosomes regulates maturation and fate of retrograde TrkA<sup>+</sup> endosomes.**

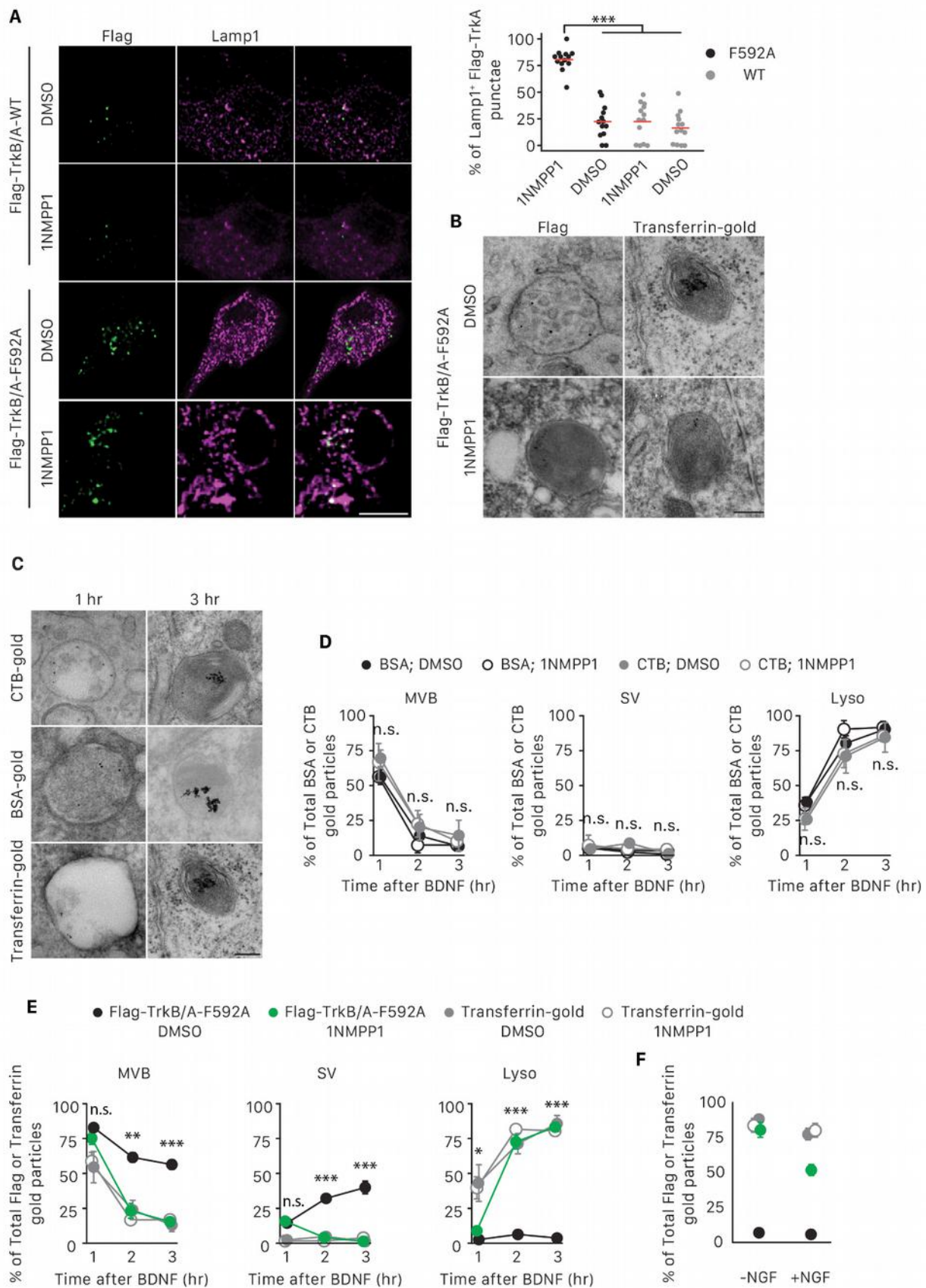
(A) Compartmentalized WT sympathetic neurons were infected with a virus expressing either Flag-TrkB/A-WT or Flag-TrkB/A-F592A. The pulse-block kinase assay was performed with BDNF instead of NGF stimulation in distal axons and with the cell body compartment treated with DMSO or 500 nM 1NMPP1 during the course of the experiments. Cells were fixed 5 hr post BDNF application, and colocalization between Flag-TrkA (green) and the lysosome marker Lamp1 (magenta) was assessed (n = 3). Scale bar: 10  $\mu$ m.

(B, E) The pulse-block kinase assay was performed in sympathetic neurons expressing Flag-TrkB/A-F592A using pre-conjugated anti-Flag antibody with Protein A-5 nm gold or a transferrin-gold tracer (6 nm). Cells were fixed at indicated time points and the number of Flag-TrkB/A-F592A or transferrin-gold particles (Tf) associated with MVBs, SVs and lysosomes was scored (E) (n = 3). Shown in (B) are representative EM micrographs of retrogradely transported Flag-TrkA and transferrin-gold in MVBs or lysosomes at the 3 hr time point. Scale bar: 100 nm.

(C, D) The pulse-block kinase assay was performed in sympathetic neurons expressing Flag-TrkB/A-F592A using CTB-gold, BSA-gold or transferrin-gold (6 nm). Cells were fixed at indicated time points and the number of gold particles associated with MVBs, SVs and lysosomes was scored for each condition (D) (n = 3). Shown in (C) are representative EM micrographs of retrogradely transported CTB-gold, BSA-gold and transferrin-gold in MVBs or lysosomes. Scale bar: 100 nm.

(F) The pulse-block kinase assay was performed as in (B) with either the presence or absence of NGF in the cell body compartment during the course of experiments. The extent of lysosomal Flag-TrkA or transferrin-gold was assessed 3 hr post BDNF application (n = 3).

Data are presented in dot plot (**A**) or represented as mean  $\pm$  SEM (**D-F**). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  by one-way ANOVA followed by Tukey's *post-hoc* correction.



## **Chapter 6: Conclusions and Discussion**

The type of endosome that mediates long-range retrograde neurotrophin signaling has been controversial. Here, we report that in both sympathetic neurons and TrkA<sup>+</sup> primary sensory neurons, multi-vesicular bodies (MVBs), not early endosomes, mediate retrograde NGF/TrkA transport and signaling. Rab7, a key MVB regulator, mediates retrograde TrkA transport and signaling both *in vitro* and *in vivo*. Moreover, our findings reveal a remarkable ‘Trojan Horse’ mechanism; TrkA complexes, which are sequestered within intraluminal vesicles of MVBs during retrograde axonal transport, become liberated within cell bodies via the formation of a novel form of single membrane vesicles (SV), rendering the receptors accessible to cytoplasmic effectors for growth and survival signaling. This MVB-to-SV transformation in cell bodies is dependent on TrkA’s catalytic activity and does not occur for MVBs carrying other ‘inert’, or non-signaling cargoes. Therefore, unlike the well-characterized role of MVBs in cargo degradation, retrogradely transported TrkA<sup>+</sup> MVBs are uniquely not degradative and capable of supporting growth factor receptor signaling. Our findings show that MVBs are dynamic, versatile structures that, depending on the nature of their cargoes, function in cargo transport, sorting, degradation and signaling.

### **The vesicular nature of retrograde signaling endosomes**

Our findings are most consistent with earlier *in vitro* and *in vivo* studies in which radiolabeled NGF was found in MVB/lysosomal structures in axons and cell bodies of sympathetic neurons (Claude et al., 1982b; Sandow et al., 2000). Although those observations implicated MVBs in mediating axonal transport of NGF, it had been unclear whether MVBs carry active TrkA receptors derived from distal axons since NGF can also bind to p75<sup>NTR</sup>, and NGF may be separated from TrkA and p75<sup>NTR</sup> following internalization. As such, whether MVBs transmit essential retrograde NGF signals within cell bodies was unknown. Our Flag-TrkA

labeling strategy provided a means to visualize retrograde TrkA endosomes, and Flag/P-Trk and Flag/P-PLC $\gamma$  double immuno-EM experiments clearly revealed a signaling competent state of retrograde TrkA<sup>+</sup> MVBs in cell bodies. This observation is in agreement with a previous study in which P-Trk immunoreactivity was found associated with multivesicular structures in the sciatic nerve (Bhattacharyya et al., 2002).

The present findings revise a long-held view in neuronal cell biology, that Rab5<sup>+</sup> early endosomes mediate long-range propagation of target-derived neurotrophic factor signals from distal axons to cell bodies. Prior evidence supporting the early endosome model included the finding of co-localization of NGF and Rab5 in neuronal soma *in vivo* and the presence of TrkA in Rab5<sup>+</sup> endosomes within cytoplasmic contents eluted from proximal segments of an *ex vivo* sciatic nerve preparation following NGF injection into the periphery (Delcroix et al., 2003). However, the lack of means for specific labeling of distal axon-derived TrkA<sup>+</sup> endosomes may complicate interpretations of those findings because of the possibility that the observed Rab5<sup>+</sup> structures were formed locally at the plasma membrane of the axon or cell body and therefore not representative of retrogradely transported TrkA<sup>+</sup> endosomes. On the other hand, we cannot exclude the possibility that early endosomes carry a small subset of retrograde NGF signals, because ~7% of retrograde TrkA endosomes observed in the pulse block experiments at the earliest time point following transport blockade are single-membrane vesicles (Figure 5E). Nevertheless, considering the differences between the quantity of TrkA<sup>+</sup> MVBs and TrkA<sup>+</sup> early endosomes, the amount of TrkA receptors carried per endosome, and differences in MVB and early endosome kinetic properties, MVBs provide a favorable means of long-range TrkA translocation and signal propagation, compared to early endosomes. It is noteworthy that, like TrkA, transferrin, BSA and CTB internalized in distal axons were also retrogradely transported

to cell bodies mainly, if not entirely, via MVBs, suggesting to us that MVBs are the predominant and possibly sole retrograde axonal transport carrier of cargoes internalized in distal axons (Figure 7C; Figure S6F).

Our *in vivo* findings demonstrate that Rab7 participates in TrkA signaling, survival and synaptogenesis of sympathetic neurons. *In vitro*, Rab7 is associated with, and necessary for retrograde transport of TrkA<sup>+</sup> signaling endosomes. Moreover, Rab7 is required for neuronal survival when NGF is applied exclusively to distal axons. The sympathetic neuron survival defect in Rab7 mutant SCGs *in vivo* is, however, less dramatic than that in SCGs of either *TrkA* or *NGF* null mice *in vivo* (Crowley et al., 1994; Smeyne et al., 1994). This discrepancy may be attributed to the incomplete ablation of *Rab7* between E14 and P0 (Figure 3.1A). Alternatively, a fraction of NGF survival signaling may occur via Rab7-independent MVB transport mechanism, NGF acting directly on cell bodies, or through early endosomes or another retrograde signal carrier that compensates for loss of Rab7 at embryonic times. Although a more complete depletion of Rab7 was achieved at P7 (Figure 3.1A), sympathetic neurons are less dependent on NGF signaling for survival at this postnatal period. Ablation of Rab7 postnatally, during a period of NGF-dependent synaptogenesis, resulted in a dramatic loss of synaptic structures in sympathetic ganglia, showing that Rab7 is required for transmitting retrograde NGF synaptogenic signals. The importance of Rab7 for retrograde TrkA transport, survival and synapse formation provides strong mechanistic support for the notion that MVBs are major carriers of retrograde NGF signals.

### **Multivesicular bodies as sorting and signaling platforms in neuronal soma**

A long-standing view is that MVBs either repress signaling via receptor downregulation or, in some cases, facilitate signaling by degrading negative regulators (Katzmann et al., 2002;

Taelman et al., 2010). Our findings provide evidence that MVBs can promote growth factor signal transduction directly. A signaling role for MVBs is supported by the presence of P-Trk tyrosine residues and P-PLC $\gamma$  associated with retrograde TrkA<sup>+</sup> MVBs in cell bodies. A major issue with respect to MVBs as signaling platforms is that the majority of TrkA signaling domains face the luminal side of the intraluminal vesicles. Thus, while the architecture of MVBs provides a plausible means of signal protection during long-range transport because of restricted access of cargo signaling domains to antagonizing and degrading cytoplasmic signals, the very same advantage imposes a conundrum as to how TrkA receptors on intraluminal vesicles can transduce RTK signals from an inaccessible location.

Our pulse-block EM experiments revealed the emergence of a new type of endosome that has a single membrane structure that does not associate with Rab5 and that exhibits a topology of TrkA receptors in which the kinase domain is oriented towards the cytoplasm and thus poised for RTK signaling within the cytoplasm. This, in combination with TrkA receptors localized to the limiting membrane of MVBs, provides a plausible means of recruitment and activation of downstream effector proteins during retrograde signaling. TrkA complexes associated with SVs are likely to derive, at least in part, from MVB ILVs since TrkA localization in this compartment dramatically decreases within minutes to hours of their appearance in cell bodies (Figure 4.2). Therefore, we propose a “Trojan Horse” mechanism for retrograde NGF signaling in which TrkA complexes, sequestered within MVB ILVs during retrograde transport, are liberated within cell bodies via the formation of SVs, rendering them accessible to cytoplasmic effectors.

To our knowledge, an MVB-to-SV maturation step has been observed in only two other examples. First, in naive dendritic cells, MHC II receptor proteins are stored within intraluminal compartments of MVBs. Upon stimulation, these receptors can translocate from MVBs to the



plasma membrane via an endosome intermediate (Chow et al., 2002; Kleijmeer et al., 2001). In a second case, Ebola virus, upon cellular entry, becomes enriched in Rab7<sup>+</sup> late endosomes and this is an obligatory step for successful infection (Saeed et al., 2010; Spence et al., 2016). The virus can later exit late endosomes and re-enter the cytoplasm. The virus is not sorted into ILVs, but its envelope, a single-pass membrane structure, is thought to fuse with the MVB limiting membrane through Niemann–Pick disease, type C proteins NPC I (Carette et al., 2011). These studies, together with the present work, indicate that MVBs are dynamic, versatile structures that, depending on the nature of their cargoes, function in cargo transport, sorting, degradation, and signaling.

### **TrkA signaling directs the maturation and fate of retrogradely transported MVBs**

While the existence of signaling endosomes following receptor endocytosis is well documented, mechanisms that underlie their maturation and metabolism have remained unclear, as has the significance of receptor activity and signaling from endosomal platforms in general (Sorkin and von Zastrow, 2002). This is at least partly due to the close spatial and temporal proximity between endosomal signaling events and signals emanating from the plasma membrane, which are difficult to distinguish in cells with a simple morphology. Studies of target-derived growth factor signaling in neurons provide a unique opportunity to address mechanisms of endosome maturation, metabolism and signaling because the plasma membrane of distal axons is physically well separated from retrogradely transported endosomes that reside within the soma. Our pulse-block paradigm, which takes advantage of the separation between the site of plasma membrane signaling and endosome formation (distal axons) and a distant site of endosome signaling, maturation and degradation (cell bodies), allowed us to monitor stages of TrkA<sup>+</sup> endosome maturation and degradation in cell bodies in a synchronized fashion. Our

findings that TrkA<sup>+</sup> SVs emerge from MVBs within cell bodies and that delayed lysosomal sorting occurs for retrograde TrkA<sup>+</sup> MVBs, but not transferrin-, BSA-, or CTB-containing MVBs, suggest that the emergence of SVs from MVBs is under the control of some unique feature of TrkA<sup>+</sup> MVBs. Indeed, we found that inhibition of TrkA kinase activity in cell bodies using a selective chemical genetic approach caused TrkA<sup>+</sup> MVBs to behave similarly to transferrin<sup>+</sup>, BSA<sup>+</sup>, and CTB<sup>+</sup> MVBs, which rapidly sort to lysosomes. Therefore, active TrkA signaling is required for the emergence of TrkA<sup>+</sup> SVs from MVBs and the prevention of precocious lysosomal sorting, both of which promote further TrkA signaling.

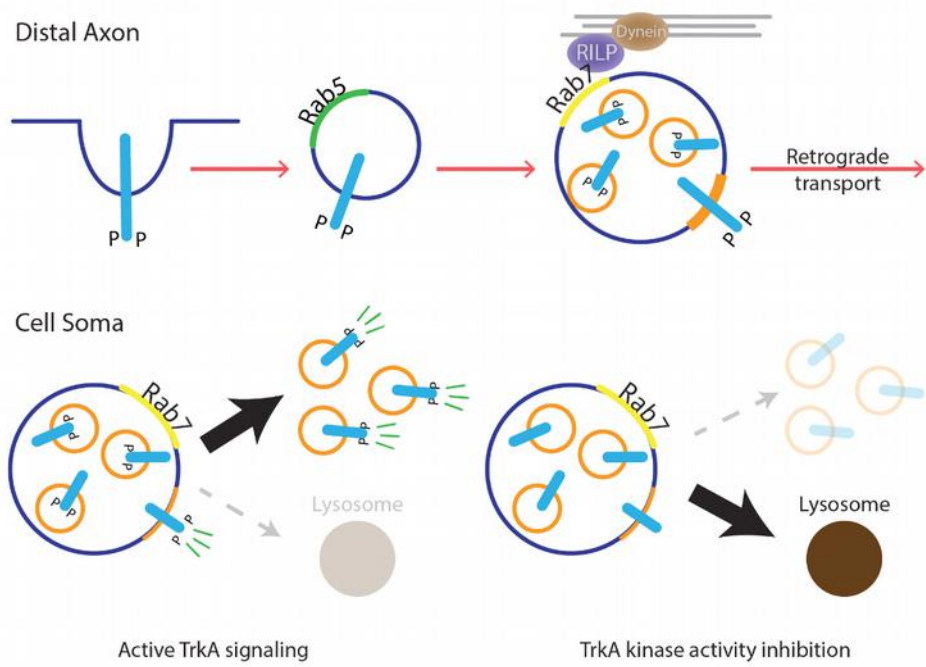
One effector protein preferentially associated with TrkA<sup>+</sup> endosomes in cell bodies, coronin, has been implicated in delaying lysosomal degradation of TrkA and promoting survival of sympathetic neurons (Suo et al., 2014). We propose a TrkA signaling-dependent, endosome-autonomous, feedforward mechanism that augments and sustains retrograde NGF signal transduction within cell bodies, through TrkA<sup>+</sup> MVB and SV recruitment of coronin and other TrkA effectors.

Taken together, our findings reveal that MVBs mediate long-range axonal transport of active TrkA, MVBs function in the cell soma as signaling and sorting platforms that give rise to signaling competent SVs, and the nature of MVB cargoes dictates MVB function and fate. It is noteworthy that defects in endosome function in neuronal processes and soma have been implicated in neurodegenerative diseases and disorders with neurological manifestations (Cosker and Segal, 2014; Harrington and Ginty, 2013). Moreover, dysfunction of early endosome activity has been implicated in several types of neurological disorders (Cooper et al., 2001; Israel et al., 2012; Salehi et al., 2006). Our findings of a central role of MVBs in retrograde neurotrophin

signaling suggest a need to evaluate potential contributions of dysfunctional MVB biogenesis, trafficking, sorting, and signaling to neurological diseases.

**Figure 6.1. Model for MVB-mediated retrograde TrkA transport and signaling.**

This schematic illustrates how multivesicular bodies control propagation of retrograde NGF-TrkA signals. In distal axons, newly internalized NGF/TrkA complexes are sorted into Rab5<sup>+</sup> early endosomes, and TrkA<sup>+</sup> early endosomes mature to form multivesicular bodies. Rab7, which localizes to TrkA<sup>+</sup> MVBs, facilitates rapid long-range retrograde axonal transport, and in turn, neuronal survival and synapse formation. In neuronal soma, active endosomal TrkA signaling induces *de novo* formation of single-membrane vesicles from retrogradely transported MVBs and prevents TrkA<sup>+</sup> MVBs fusion with lysosomes. Together, these activity-dependent MVB dynamics promote and sustain transduction of retrograde NGF signals. This model highlights critical roles of MVBs in a series of events during target-derived neurotrophic factor signaling in neurons.



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# Curriculum Vitae

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